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Dietary Production of Gastric Ulcers in Rats and Prevention by Tocopherol Administration.

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A previous paper[1] from this laboratory reported that α-tocopherol prevented stomach ulcers in rats receiving minimal amounts of vitamin A. The possible explanation advanced to explain this action was based upon the well-known "sparing" of vitamin A by tocopherol, *in vivo*.[2]

Further experiments were designed to test this explanation and also to determine whether tocopherols would be beneficial in preventing stomach ulcers caused by dietary restrictions other than vitamin A.

In a preliminary experiment 40 weanling male rats were placed on a diet (USP XII, with olive oil), low in vitamins A and E, for 2 weeks. At this time supplementation was begun of all rats with 300 units of vitamin A-acetate daily. Half of the rats were also given 0.5 mg of d,α-tocopherol daily. After 7 weeks the animals were killed and examined for gastric lesions. Fifty per cent of the rats on the high-A, low-E diet had one or more ulcers in the fore-stomach, while none of the rats receiving vitamin E had lesions.

In a second experiment 170 male weanling rats were completely depleted of vitamin A in the usual manner. The diets used were based upon the USP vitamin A-test diet, but with variable amounts and kinds of fat. These variations are indicated in Table I. At the time of depletion 3 or 4 rats from each group

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[*] Communication No. 108.

[1] Jensen, J. L., *Science*, 1946, **103**, 586.

[2] Hickman, K. C. D., Kaley, M. W., and Harris, P. L., *J. Biol. Chem.*, 1944, **152**, 303; Harris, P. L., Kaley, M. W., and Hickman, K. C. D., *J. Biol. Chem.*, 1944, **152**, 313; Hickman, K. C. D., Kaley, M. W., and Harris, P. L., *J. Biol. Chem.*, 1944, **152**, 321.

TABLE I.

Effect of Tocopherol and Dietary Fat on Stomach Lesions of Rats After Cure of Vitamin A Deficiency with High Doses of Vitamin A (10 Rats per Group).

			Supplement after depletion				
Group	Diet—Fat	Avg depletion time (days)	Units Vit. A per day	Tocopherol per day	Avg wt gain 48 days	Incidence of gastric ulcers, %	Avg severity of ulcers graded 1-10
I	5% olive oil	62	30		96.4	50	4.6
I_A	” ” ”	62	30	.5 mg *d,α*	94.0	0	—
I_B	” ” ”	62	2		56.9	60	5.1
I_C	” ” ”	62	2	” ” ” ”	63.1	0	—
I_D	” ” ”	62	2	” ” phosphate inj.	59.8	50	4.3
II	10% ” ”	65	30		97.3	0	—
II_A	” ” ”	65	30	.5 mg *d,α*	100.7	0	—
III	5% stripped corn oil	60	30		99.1	30	3.7
III_A	” ” ” ”	60	30	” ” ” ”	101.9	0	—
IV	10% ” ” ”	61	30		98.4	20	2.0
IV_A	” ” ” ”	61	30	” ” ” ”	100.2	0	—
V	Fat-low	51	30		62.5	80	6.1
V_A	” ”	51	30	” ” ” ”	57.8	80	5.7
V_B	” ” + 100 mg sesame oil	51	30		77.1	30	3.9
V_C	” ” ” ” ” ” ”	51	30	” ” ” ”	83.8	10	2.3

were examined, but no gross lesions were present. It is evident that vitamin A-deficiency *per se*, does not produce ulcers. The depleted animals were placed on a daily supplement of 2 or 30 units of vitamin A-ester concentrate, dissolved in olive oil, with or without 0.5 mg α-tocopherol. Half of the rats on the low-fat diet were supplemented daily with 100 mg of sesame oil to supply adequate linoleic acid.

Table I shows the data on gain in body weight and incidence and severity of rumen lesions after a 7-week experimental period.

It is highly significant that vitamin A even in high doses did not prevent ulcer development. This indicates that the action of tocopherol cannot be explained simply a sparing of vitamin A in the gastrointestinal tract. However, the fact that injections of tocopheryl phosphate did not prevent the lesions is presumptive evidence that the tocopherol effect is localized in the stomach. It is interesting to reflect at this time that in all vitamin A bioassays which have been conducted without providing adequate vitamin E to the animals during the depletion or curative periods, 50% or more of the animals used probably had gaping lesions in their stomach. These gastric ulcers certainly were an important factor in causing some of the disturbingly wide variations in growth response observed in vitamin A bioassays.

The influence of fat in the diet is interesting. The lesions occurred on the 5% level of olive oil or stripped (E-free) corn oil; but on the 10% oil level the lesions were absent or reduced in number even without tocopherol supplements. On the low-fat diet a very high incidence and severity of lesions was noted and tocopherol at the levels used had no beneficial effects.

Since fat in the diet is a significant factor in the prevention of rumen lesions, it was of interest to examine the stomachs of rats deficient only in essential fat acids. The fat-low diet used in the preceding experiment contained 56% corn starch which probably supplied about 0.6% fat as a constituent.[3] Thus the diet contained about 0.3 to 0.4% fat which furnished at least 10 mg linoleic acid daily to each animal.[4]

In the present experiment, weanling rats were placed on a fat-free diet of the following percentage composition: Casein (Labco) 20, sucrose 76, and salt mixture, USP No. 2, 4. B-complex vitamins were added to the casein to furnish 10 γ of thiamin, riboflavin, and pyridoxine, 25 γ calcium pantothenate, 1 mg choline chloride, and 0.1 mg *i*-inositol per gram of ration. Fifty units of vitamin A-acetate and 5 units of vitamin D were given daily in a propylene glycol solution.

After 7 weeks depletion the rats were divided into groups of 6 each and placed on experiment with and without 20 mg of ethyl linoleate also with and without 0.5 mg tocopherol daily. After 6 weeks the stomachs of the rats were examined for lesions. The results are shown in Table II. Hyperplasia of the juncture (or ridge) separating the glandular portion from the rumen, usually precedes or accompanies hyperplastic ulcers in the rumen. The incidence of such juncture hyperplasia has been indicated, as well as the incidence of ulcers.

The data in Table II show that rats deficient in essential fatty acids do not have stomach lesions. However, half of the rats receiving 20 mg of linoleate showed typical ulcers. Juncture hyperplasia was marked in this group. Either α- or γ-tocopherol prevented the lesions.

It was of further interest to determine whether ulcers produced by dietary restrictions other than vitamin A or essential fatty acids would also be prevented by tocopherol.

Male albino weanling rats were placed upon a pyridoxine-deficient ration for 6 weeks. The percentage composition of the ration was: Casein (Labco) 20, sucrose 71, salt mixture, USP No. 2, 4, lard 4, and cod liver oil 1. B-complex vitamins were added to the casein to furnish 10 γ thiamin and riboflavin, 25 γ calcium pantothenate, 1 mg choline chloride, and 50 γ of vitamin K per gram ration.

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[3] Taylor, T. C., and Nelson, J. M., *J. Am. Chem. Soc.*, 1920, **42**, 1726.

[4] Taylor, T. C., and Lehrman, L., *J. Am. Chem. Soc.*, 1926, **48**, 1739.

TABLE II.
Effect of Tocopherol and Methyl Linoleate on Development of Gastric Lesions in Rats Deficient in Essential Fatty Acids.*

	Daily supplement		Incidence of gastric lesions	
Trial	Methyl linoleate (mg)	d,α-tocopherol (mg)	Ulcers (%)	Juncture hyperplasia (%)
I	0	0	0	0
	0	0.5	0	17
	20.0	0	50	67
	20.0	0.5	0	0
	20.0	0.5†	0	0
II	0	0	0	33
	20.0	0	0	83
	20.0	0.5	0	17

* 6 animals per group.
† γ-tocopherol.

TABLE III.
Effect of Tocopherol in Preventing Development of Rumen Lesions in Rats Recovering from Pyridoxine Deficiency.

Pyridoxine (γ daily)	Mg α-tocopherol daily	No. of rats	Avg wt gain in 6 wk (g ± S.D.)	Incidence of rumen lesions (%)
0	0	7	4.3	0
	1	7	7.1	0
1.0	0	4	51.5 ± 6.3	50
	1	4	53.5 ± 4.9	0
2.0	0	7	71.4 ± 7.6	57
	1	7	82.7 ± 8.3	0
4.0	0	7	91.3 ± 5.0	29
	1	7	105.8 ± 4.8	0
8.0	0	6	124.0 ± 7.7	0
	1	5	120.0 ± 6.0	0

After 6 weeks depletion, the rats were fed various levels of pyridoxine with and without tocopherol. Pyridoxine (free-base) was prepared from synthetic pyridoxine-hydrochloride (Merck) and given in propylene glycol solution from a calibrated dropper.

The resulting gains in body weight during the subsequent 6 weeks and the incidence of rumen lesions at this time are summarized in Table III. These data indicate that pyridoxine deficiency in itself does not result in rumen lesions. However, when suboptimal doses of pyridoxine are fed, lesions do occur but are preventable by α-tocopherol. Higher doses of pyridoxine maintain normal stomachs in the rats even without tocopherol.

Discussion. It is evident that gastric lesions can be induced by a variety of dietary deficiencies when these are combined with low intakes of vitamin E. Severe tocopherol deficiency uncomplicated by deficiencies of other essentials does not induce ulcers. Hundreds of vitamin E-deficient female rats with evidence of resorption gestations have been free of stomach lesions. Furthermore, uncomplicated deficiencies of vitamin A, or of pyridoxine, or of essential fatty acids do not induce ulcers. Ulcers are formed only in rats which have stopped gaining weight as a result of any of these deficiencies and which then have been given the missing factor but without supplying tocopherols at the same time. Weight gain is resumed when vitamin A, pyridoxine, or linoleate, respectively, is supplied and it is during this period that stomach lesions appear unless sufficient tocopherol is present in the gastrointestinal tract. Tocopherols injected as the tocopheryl phosphate during the experimental period fail to prevent ulcer formation. Consequently, the evidence points to a mechanistic explanation for lesion formation. Functionally, the epithelium of the forestomach of the rat becomes abnormal due to deficiencies of either vitamin A, or pyridoxine, or essential fatty acids. Anatomically there are no lesions because of the gradual adaptation of the organism as a whole to a low level of metabolic activity: food intake decreases, weight gain ceases, and probably gastric secretion of pepsin and HCl is diminished. However, administration of the missing essential factor induces food intake and resumption of weight gain. Unless adequate tocopherol is also administered the functionally abnormal epithelium of the forestomach cannot cope with the increased acid-pepsin medium with which it is in contact and as a result lesions are produced.[5] Whether tocopherols prevent the erosion of the stomach wall by specifically inhibiting enzyme activity or more fundamentally by increasing epithelial resistance perhaps by inducing greater mucous secretion or improving blood circulation in the mucosa[6] is not yet known.

The role of dietary fat in the etiology and treatment of gastric lesions is also undetermined. In our experiments an increase of fat (olive oil or vitamin E-free corn oil) from 5 to 10% in the diet reduced the incidence and severity of ulcers formed. Li and Freeman[7] also found a higher incidence of peptic ulcers in dogs on a low-fat (lard) than on a high-fat diet. However, Matzner and coworkers[8] reported their highest incidence and most severe stomach lesions in rats maintained on relatively high fat (butter or lard) intakes.

It is emphasized that no attempt should be made at this time to draw a parallel between these gastric ulcers in rats and those in humans. Much more must be learned concerning the etiology and pathology of the lesions in the 2 species before generalizations regarding treatment with fat or tocopherols can be made. The level of protein intake is also important and data showing a protein-tocopherol interrelationship as measured by ulcer production and cure is being prepared for publication.

Conclusions. 1. Ulcers in the forestomach were induced in rats: (a) Recovering from vitamin A-deficiency with 2, 30, or 300 units vit. A daily. (b) Recovering from a vit. B_6 deficiency with suboptimal amounts (1, 2, or 4 γ daily) of pyridoxine. (c) Recovering from an essential fatty acid deficiency with 20 mg of linoleate daily.

2. Deficiencies of these 3 essentials did not, in themselves, result in lesions. The ulcers occurred only during the curative period in which the specific nutrient was fed.

3. α-Tocopherol fed daily to the rats during the cure of the specific deficiency gave complete protection. γ-Tocopherol orally was also effective, but α-tocopheryl phosphate injected was not.

4. The amount of fat in the diet was related to the incidence of stomach ulcers in the vitamin A experiments. Ten per cent gave good protection. Five per cent allowed moderately severe lesions, which were preventable by tocopherol. Fat-low diets gave very severe lesions not preventable by the level of tocopherol fed, 0.5 mg daily.

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[5] Matzner, M. J., Windmer, C., Sobel, A. E., and Polayes, S. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 243.

[6] Bachrach, W. H., Grossman, M. I., and Ivy, A. C., *Gastroenterology*, 1946, **6**, 563.

[7] Li, T., and Freeman, S., *Gastroenterology*, 1946, **6**, 140.

[8] Matzner, M. J., Windmer, C., and Sobel, A. E., *Am. J. Dig. Dis.*, 1938, **5**, 1.

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Nature of Renal Changes in Acute Choline Deficiency.

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Changes in the kidneys of acutely choline-deficient young rats, referred to as hemorrhagic degeneration of the kidneys were described by Griffith and Wade,[1] and later by Györgyi and Goldblatt,[2] Christensen,[3] Engel and Salmon[4] and others. The most careful histological study by Christensen showed the quite secondary role of hemorrhage in this syndrome, but left open the question of interdependence of tubular degeneration and vascular congestion.

Experimental. Young rats, 20 to 21 days old and weighing about 35 g, were placed in individual cages and fed water and the following diet *ad libitum:*

Basal diet:

	%
Sucrose	65
Alc. Ext. Casein	15
Salts No. 2	5
Lard	10
Gelatin	3
Corn Oil	2

with the addition of the following vitamins/kg:

Thiamin chloride	10	mg
Pyridoxine hydrochloride	10	''
Calcium pantothenate	20	''
Niacin	10	''
Para-aminobenzoic acid	20	''
Riboflavin	10	''
Inositol	50	''
Biotin	0.2	''
Vit. E	25	''
Menadione	5	''
Vit. A	20,000	units
'' D	2,000	''

It was determined by observation that under our conditions the renal syndrome began to appear at about the fifth day. Thus most experiments were terminated on the seventh day since by this time all the rats would show marked renal hyperemia.

A detailed examination of the kidneys for structural changes due to this deficiency, led to the following conclusions as to the nature and sequence of these changes:

(1) Both grossly and microscopically the change observed first is venous stasis resulting in extreme cyanosis of the outer cortex.

(2) Absence of red blood cells from the lumen of the tubules is evidence of lack of hemorrhage.

(3) Epithelial degeneration and necrosis, limited to the tubules occupying the outer cortex, are secondary to the vascular disturbance.

(4) The lack of glomerular involvement, in sharp contrast to the marked tubular injury, proves interference with venous outflow rather than disturbance of arterial supply.

The outlined sequence of events of the renal syndrome is observed in reversed order in the phase of recovery, with venous stasis disappearing most rapidly, epithelial regeneration following considerably later, and hyaline casts, distended tubules and small scars forming the last residues of the disturbance. That interference with venous outflow is involved, finds support in our observations, that ligation of the renal vein in rats results in lack of damage to the glomeruli while there is marked injury to the tubular epithelium.

Experimental evidence of the priority of vascular disorder over epithelial injury is suggested by the protective effect of renal decapsulation. This operation was performed under ether anesthesia on the left side only, the right kidney serving as a control. In one group of experimental animals an incision was made on both sides but decapsulation of the kidney was performed on the left side only. The operation took place on the day that the rats were put on the choline-deficient diet. This operation gave protection to the decapsulated kidney in proportion to the completeness of the operation. In 19 out of 34 animals there was complete protection from the renal syndrome of acute choline deficiency on the operated side. This protection seems to be in abeyance if the operation is not performed well in advance of the acute disturbance, since if the decapsulation is not done until the fifth day, no protection is observed.

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[1] Griffith, W. H., *Biol. Symposia*, 1941, **5**, 193; Griffith, W. H., and Wade, N. J., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 188.

[2] Györgi, P., and Goldblatt, H., *J. Exp. Med.*, **1940**, **72**, 1.

[3] Christensen, K., *Arch. Path.*, 1942, **34**, 633.

[4] Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

Wolbach[5] has suggested the possibility that a neurovascular mechanism might be the basis of the renal syndrome observed in acutely choline-deficient rats. There is support for such an assumption in our findings that subcutaneous administration of atropine seems to inhibit the renal syndrome. When rats were treated with 1 mg atropine sulfate daily, from the day they were started on the choline-deficient diet, prevention of the renal syndrome was observed in almost half of the cases. In these preliminary experiments, which lasted 7 days, 10 of 21 choline-deficient young rats failed to show the "hemorrhagic renal syndrome."

Summary and Conclusion. Morphological and experimental studies suggest a vascular disorder to be the primary injury observed in the kidney of the acutely choline-deficient rat.

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[5] Wolbach, S. B., and Besser, O. A., *Physiol. Rev.*, 1942, **22**, 233.

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Reticulo-Endothelial Immune Serum (REIS). VI. Production of Potent Serum by Anamnestic Reaction.*

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The development of antiorgan sera has been reported in studies from our laboratories.[1] Whereas in our original work[2] we followed the method of Soviet workers,[3] some modifications in the production of REIS were introduced in the present study. It was thought that an accumulation of antibodies in the recipient host would be enhanced by a slow process of immunization rather than by the rapid method of using massive doses.[4]

In the preparation of antiguinea pig and of antirat sera, constant amounts of tissue, 400 mg of spleen plus 100 mg of bone marrow, suspended in 6 ml of saline were administered to the rabbit. Intravenous antigen injections of 0.25, 0.5, 0.5, and 0.75 ml were given at 4-day intervals, and the same amounts were given by mouth, the total being approximately 133 mg of spleen and 33 mg of bone marrow by each route within 12 days.

Complement-fixation titres illustrate the high values of antirat and antiguinea pig sera following low antigenic dosages.

The achievement and maintenance of adequate potency of the serum was one of the main objectives. Attempts were made, therefore, to provoke an increased antibody production in previously immunized animals by their anamnestic response to a renewed contact with the antigen. A single intravenous injection of the same type of antigen 30-40 days following the last injected dose was administered to rabbits.

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* Supported by a grant from the Lilly Research Laboratories.

[1] Pomerat, C. M., *Phi Beta Pi Quart.*, 1945, **42**, 203.

[2] Pomerat, C. M., and Anigstein, L., *Tex. Rep. Biol. and Med.*, 1945, **3**, 122.

[3] Marchuk, P. D., *Am. Rev. Sov. Med.*, 1943, **1**, 113.

[4] Straus, R., Runjavac, M., Zaitlin, R., Duboff, G., and Swerdlow, H., *II. J. Immun.*, 1946, **56**, 155.

TABLE I.
Showing Comparative Values of REIS Potency in a Unit Experiment.

Rabbit No.	Antigen source	REIS complement-fixation titers Primary	REIS complement-fixation titers Anamnestic	Spleen explant outgrowth in REIS 1:4 Primary	Spleen explant outgrowth in REIS 1:4 Anamnestic
68	Chick	1:60	X	+	X
69	ʼʼ	1:40	1:640	++	+
70	Guinea pig	1:640	1:1280	+++	+
71	ʼʼ ʼʼ	1:1000	1:2000	+	0
72	Rat	1:1000	X	0	X
73	ʼʼ	1:320	1:160	+	++

X Animal sacrificed.
0 No outgrowth—complete inhibition.
+ Slight outgrowth.

The serum of rabbit 69 (Table I), treated with chick antigen, primarily showed a titre of only 1:40. This rabbit was given a booster dose 30 days later of 1:25 ml suspension of 400 mg of chick spleen and 100 mg of bone marrow in 6 ml saline. The animal was bled 9 days later and at this time the "anamnestic" serum, as compared with the "primary," showed a titre for complement fixing antibodies of 1:640. A 100% rise in the titre was obtained in 2 other rabbits previously treated with guinea pig antigen. On rare occasions there was no response to the booster method (rabbit 73).

The potency of the serum was also evaluated by the tissue culture assay method. It was previously suggested[2,5] that it is possible to measure the action of antiorgan preparations by the degree of inhibition of cellular outgrowth from explants in a medium containing various concentrations of REIS. The outgrowth equivalent to that of untreated explants was designated as ++++. When the serum of rabbit 69 (primary titre 1:40) was tested, the outgrowth of 15- to 17-day chick embryonic spleen fragments was moderately good (++), showing weak inhibitory effect of the serum. However, the serum of the same rabbit, after the anamnestic reaction, reaching the titre of 1:640 almost completely inhibited the outgrowth. Marked clumping of outwandering cells under the influence of the serum of increased potency at dilutions of 1:4, 1:8, and 1:16 was noted. The anamnestic serum totally inhibited the outgrowth in concentrations of 1:4, 1:8, 1:16, 1:32 as compared with a slight inhibition of the primary serum.

Summary. In the preparation of antiorgan sera the administration of small amounts of antigen (spleen and bone marrow) was found more effective than the rapid immunization of rabbits with massive antigen dosage. A single injection of the antigen 30-40 days after the last dose gives rise to an anamnestic response of the host and to a serum of higher potency. This can be evaluated by complement fixation titres and by the outgrowth of tissue explants exposed to the action of the "anamnestic" serum.

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[5] Pomerat, C. M., and Anigstein, L., *Science*, 1944, **100**, 456.

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Effects of Benadryl on Anaphylactic and Histamine Shock in Rabbits and Guinea Pigs.

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Recent work[1,2] suggests that the mechanism of anaphylactic shock is best explained with the inclusion of a stage at which histamine is released. On the assumption that the release of histamine is the factor leading to shock and death, therapeusis of anaphylactic and allergic states has turned to β-dimethylaminoethyl benzhydryl ether hydrochloride ("Benadryl") and related drugs.[3,4] Success in this has been offered as further proof of the thesis that histamine intoxication is the chief untoward effect of anaphylaxis. Protection against anaphylaxis by benadryl was reported in experiments on passive sensitization of guinea pigs and intravenous administration of the antigen.

The experiments here reported resulted from a search for the antianaphylactic effects of benadryl. In the 2 species studied, one might expect that if the principal symptoms of shock and the cause of death attendant on anaphylactic shock were ascribable to the release of histamine, the amelioration of anaphylactic shock by benadryl would parallel its effects on histamine shock. That such is not the case is shown by the experiments which follow; the implications of these findings will be discussed.

Experiment I. To test the effects of benadryl on histamine shock in rabbits, 25 animals were employed, 12 of which received premedication with benadryl. The animals fell into 3 groups, each containing rabbits of similar size and history and each being divided into experimental and control groups. Benadryl was administered in divided doses totaling 10 mg per kg or in single doses of 4 mg per kg. Striking protection at all the dose levels is demonstrated in Table I.

Experiment II. To test the effects of benadryl on anaphylactic shock in rabbits, 23 animals were sensitized to egg white. This was accomplished by injecting egg white on alternate days in the following dosages: 1 cc, i.v.; 0.5 cc, i.v.; and 1 cc, i.m. The rabbits were used between 3 and 5 weeks following the first dose. Group A consisted of 10 rabbits so sensitized, half of which were premedicated with 5 mg of benadryl per kg 25 minutes before injection of the antigen. The 5 rabbits not treated with benadryl were all severely shocked, and 4 of 5 died. Those premedicated were similarly shocked, and none of the 5 animals recovered. In group B, benadryl (5 mg per kg in 3 animals; 10 mg per kg in 3) was administered 24 hours previously, with a second dose 20 minutes before the antigen. No difference in the effects of a smaller dose of antigen (0.6 cc) was observed between experimental and control animals.

Experiment III. To test the effects of benadryl on anaphylactic shock in the actively sensitized guinea pig, each of 11 animals was sensitized by a single intraperitoneal injection of 5 cc 25% egg white in saline. Twenty days later 7 of these received 10 mg of benadryl per kg administered intraperitoneally. Thirty minutes later 5 of the premedicated animals and 4 controls were injected intraperitoneally with 0.75 cc of egg white. All animals showed severe shock; 3 of 4 control, and 3 of 5 treated, guinea pigs died. Two animals given only benadryl showed no ill effects.

Discussion. Experiment I indicates that benadryl is, under the conditions of the ex-

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* Trainee of the National Cancer Institute.

[1] Dragstedt, C. A., *J. Allergy*, 1945, **16**, 69.

[2] Code, C. F., *Proc. Staff Meetings Mayo Clin.*, 1945, **20**, 439.

[3] Loew, E. K., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

[4] Friedlaender, S., Feinberg, S., and Feinberg, A. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 65.

TABLE I.
Effects of Benadryl on Histamine Shock in Rabbits.

Group	No.	Wt, kg	Premedication	Histamine dose	Effect*
A	1	1.8	None	2.0 mg hist. phosph.	4
	2	1.8	,,	,,	4
	3	1.8	,,	,,	4
	4	1.8	,,	,,	4
	5	1.8	,,	,,	3
	6	1.8	Benadryl, i.p., 5 mg per kg 4 hr before hist., plus 5 mg per kg 15 min before hist.	,,	1
	7	1.8		,,	0
	8	1.8		,,	0
	9	1.8		Died before hist. was admin.	
B	10	2.5	None	2.75 mg hist. phosph.	2
	11	3.4	,,	4.13	4
	12	3.0	,,	3.44	3
	13	3.0	,,	2.75	4
	14	3.0	Benadryl, i.p., 4 mg per kg, 15 min. before hist.	5.50	3
	15	3.2		,,	0
	16	3.0		,,	0
	17	3.6		,,	0
C	18	2.3	None	2.10	3
	19	2.3	,,	,,	4
	20	2.3	,,	,,	4
	21	2.3	,,	,,	3
	22	2.3	Benadryl, i.p., 4 mg per kg, 15 min before hist.	2.10	2
	23	2.5		,,	0
	24	2.3		,,	0
	25	2.3		,,	2

* Four grades of shock were as follows: 1, signs of collapse but animal able to move when stimulated; 2, resting in normal position but unable to move; 3, prostrate position or on side; 4, dead of shock.

periment, an effective antagonist of histamine. This means either that it opposes histamine in its effect on the shock organs or that it acts chemically to remove or neutralize histamine. The present experiments do not differentiate between these 2 possible modes of action and the reader is referred to the recent discussion of Code.[2] The lack of antianaphylactic effect of benadryl shown in Experiment II, even in doses which were highly effective against histamine, indicates either that the histamine released upon anaphylactic shock is not vulnerable to this antihistamine drug, or that some other factor is involved in the shock and death caused by the antigen. As the most recent demonstrations of the antianaphylactic nature of these drugs have been made on guinea pigs, Experiment III on that species was set up (Table III). The 5 experimental animals all showed severe shock, though treated with 10 mg per kg of benadryl. Our results seem at direct variance with those of Loew and Kaiser[3] and Friedlaender, Feinberg, and Feinberg,[4] for they conclude that benadryl offers marked protection to anaphylaxis in the guinea pig. However, it is important to note that their animals were passively sensitized and that they administered the antigen intravenously. While the shock states which they observed were primarily pulmonary in nature, in our animals, in which the antigen was administered by a different route, the shock developed more slowly and was characterized principally by prostration with few respiratory symptoms. Similar alteration of anaphylactic shock with routes of administration other than intravenous have been demonstrated by Williamson.[5]

Summary. 1. Benadryl offers effective pro-

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[5] Williamson, R., *J. Hyg.*, 1936, **36**, 588.

TABLE II.
Effects of Benadryl on Anaphylactic Shock in Rabbits.

Group	No.	Wt, kg	Sensitive	Premedication	Shock dose	Effect
A	1	2.0	Yes	None	1.0 cc egg white	4
	2	1.8	’’	’’	’’	4
	3	1.8	’’	’’	’’	4
	4	2.0	’’	’’	’’	4
	5	1.8	’’	’’	’’	3
	6	2.3	’’	Benadryl, i.p., 5 mg per kg 25 min before injection with antigen	’’	4
	7	1.8	’’		’’	4
	8	1.8	’’		’’	4
	9	2.0	’’		’’	4
	10	2.3	’’		’’	4
B	11	1.8	’’	Benadryl, i.p., 5 mg per kg 24 hr before shock, plus 5 mg per kg 20 min before antigen.	0.6 cc	3
	12	1.8	’’		’’	2
	13	1.8	’’		’’	3
	14	1.8	’’	Benadryl, i.p., 10 mg per kg 24 hr before shock, plus 5 mg per kg 20 min before antigen.	’’	4
	15	1.8	’’		’’	3
	16	1.8	’’		’’	4
	17	1.8	’’	None	’’	3
	18	1.8	’’	’’	’’	4
	19	1.8	’’	’’	1.0 cc	3
	20	1.8	’’	’’	’’	3
	21	1.8	’’	’’	’’	2
	22	1.8	’’	’’	’’	4
	23	1.8	’’	’’	’’	2

TABLE III.
Effects of Benadryl on Anaphylactic Shock in Actively Sensitized Guinea Pigs.

No.	Wt, g	Premedication	Antigen	Results
1	350-400	None	0.75 cc egg white, i.p.	Died in 20 min.
2	’’	’’	’’	’’ ’’ 30 ’’
3	’’	’’	’’	’’ ’’ 35 ’’
4	’’	’’	’’	Survived prolonged severe shock
5	’’	10 mg per kg 30 min before antigen	’’	Died in 30 min.
6	’’	’’	’’	’’ ’’ 35 ’’
7	’’	’’	’’	’’ ’’ 40 ’’
8	’’	’’	’’	Survived prolonged severe shock
9	’’	’’	’’	’’ ’’ ’’ ’’
10	’’	’’	None	No ill effects
11	’’	’’	’’	’’ ’’ ’’

tection against histamine shock in rabbits. 2. Benadryl did not protect against anaphylactic shock in rabbits sensitized to egg white. 3. Benadryl did not protect guinea pigs actively sensitized to egg white against anaphylaxis induced by intraperitoneal administration of the antigen under the dosage conditions employed.

15770

Methionine Decholesterolization in Old Hens.*†

George R. Herrmann.

From the University of Texas, Medical Branch, Galveston, Texas.

Choline has been shown in our previous studies to have a decholesterolizing effect in old hens.[1] Methionine has been found to take part in a reversible reaction with choline in the animal body[2] and to substitute for choline[3] and be effective in lipid metabolism disturbances in the liver of mammals.[4,5] It therefore seemed logical to study methionine, just as we have choline and inositol, as a possible decholesterolizing agent in old hens with hypercholesterosis.

Forty-four 2-year-old white leghorn hens were individually caged and fed the standard Texas A & M laying mash. The initial blood lipid levels were determined by the methods of Bloor[6] for total cholesterol, Bloor and Knudson[7] for cholesterol esters, and King[8] for total and inorganic phosphorus.

Methionine, 0.5 g, was placed daily in the gullet of each of 24 of the old hens. One hen died of liver disease. After feeding the methionine for 18 to 29 days, the hens were bled a second time and the blood lipids were again determined, but, as shown in Table I, very little decholesterolization had occurred. The administration of methionine was continued, third bleedings were made after 36 to 51 days, and the bloods chemically analyzed as before.

When random samples of blood showed

TABLE I.
Effect of Methionine on Cholesterol in Blood and Tissue of Old Hens.

	Blood cholesterol, mg/100 ml		Lipid Phosphorus, mg/100 ml	Tissue cholesterol, mg/100 g					
				Aorta		Heart		Liver	
	Total	Esters		Total	Esters	Total	Esters	Total	Esters
Controls									
20 old hens	195	147	10.7	253	188	233	172	303	223
S.D.*	±18	±20	±2	±50	±47	±48	±36	±50	±38
23 old hens									
1st bleeding	228	171	11.2						
S.D.	±33	±23	±2.3						
Methionine									
2nd bleeding	171	126	14.3						
18-29 days S.D.*	±29	±28	±2.4						
3rd bleeding	138	95	16.6	156	102	135	91	276	169
36-57 days S.D.*	±25	±24	±4	±21	±21	±19	±13	±94	±63

* S.D.—Standard Deviation.

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* With the technical assistance of Anna H. Williams, Betty Ann Biel, John E. Prewett, H. Tom Leigh, and George W. Reimer.

† Methionine was generously supplied by John Wyeth Company, Philadelphia, and the study was, in part, supported by a grant from the Medical Research Department of the Winthrop Chemical Company.

[1] Herrmann, G. R., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 229, 302; **63**, 436.

[2] Simmonds, S., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **146**, 685.

[3] Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1939, **131**, 567; Griffith, W. H., *J. Nutrition*, 1940, **19**, 437.

[4] Channon, A. J., Manifold, M. C., and Eckstein, H. C., *J. Biol. Chem.*, 1940, **135**, 886.

[5] Tucker, H. P., Treadwell, C. R., and Eckstein, H. C., *J. Biol. Chem.*, 1940, **135**, 85.

[6] Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

[7] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

[8] King, E. J., *J. Biol. Chem.*, 1932, **26**, 240.

that the cholesterol levels were significantly lowered, the hens were sacrificed and blood collected for lipid analysis. The aorta, about a quarter gram of heart muscle and a similar mass of liver were individually and accurately weighed, then macerated with sand and 1 to 3 ether alcohol solution. The extract of each organ was analyzed and the amounts of total cholesterol and cholesterol esters in 100 g of each tissue calculated. Twenty old hens fed as simultaneous controls were also sacrificed and the blood and tissues likewise treated. The results are set down as controls in Table I.

It is evident that the total blood cholesterol and cholesterol ester levels definitely decreased and the phospholipids rose after the old hens had been given 0.5 g methionine daily for about 3 weeks. After 5 to 7 weeks treatment, significantly lower levels of the blood cholesterol and cholesterol esters were established, and the aorta, heart, and liver showed significantly lower lipid levels than those of the control series. Methionine therefore, seems to act as a decholesterolizing agent in old hens.

15771

Fibrinogenolytic Demonstration of Activation and Inhibition of Tryptase in Plasma Protein Fraction-I ("Antihemophilic Globulin").*

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Previous experience has shown the superiority of lysis of fibrinogen[1] over fibrinolysis[2] in the assay of tryptic enzymes. Recent revival of the idea[3] that the natural blood-clotting system involves the same protease (serum-tryptase) that is responsible for fibrinolysis[4] and other proteolytic phenomena is based upon experimental analogies with crystalline pancreatic trypsin. The ability of trypsin to restore or improve the clotting of hemophilic blood *in vitro*[5] and *in vivo*[6] suggests a plasma tryptase deficiency in hemophilia, for which not-altogether-conclusive evidence is afforded by data[7,8] on proteases demonstrable by the Delezenne and Pozerski (1903) chloroform-activation method, and by similar finding[9] of such proteases in certain "globulin" plasma protein fractions, which the Harvard investigators[10] have developed in application to the clinical treatment of hemophilia. The present observations employ a fibrinogenolytic reaction[1] to establish some fundamental considerations with respect to the development of "tryptase" activity in fibrinogen-rich human plasma fractions, as compared with similar materials of bovine plasma origin.

Reagents. 1. H.F.: human plasma Fraction I, courtesy of Drs. Cohn, Minot, Edsall, and colleagues (Harvard Medical School);[10-12]

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* This work is the first of a series of investigations on "Enzymes and enzyme-inhibitors, in relation to blood coagulation and hemorrhagic diseases," aided by a grant from the John and Mary R. Markle Foundation.

[1] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

[2] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 73.

[3] Ferguson, J. H., *Science*, 1943, **97**, 319.

[4] Nolf, P., *Medicine*, 1938, **17**, 381.

[5] Ferguson, J. H., *Am. J. Physiol.*, 1939, **126**, 669.

[6] Tagnon, H. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 45.

[7] Feissly, R., *Schweiz. Med. Wschr.*, 1942, **72**, 648.

[8] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1943, **22**, 127.

[9] Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

[10] Lewis, J. H., Tagnon, H. J., Davidson, C. S., Minot, G. R., and Taylor, F. H. L., *Blood*, 1946, **1**, 166.

TABLE I.
Clotting-times in Relation to Concentration of Plasma Fraction.*
Clotting-times (sec.) at 25°C for 0.5 cc F (strengths* cited) + 0.5 cc T (1%).

F.	Plasma fraction	Strength* of solution (% dry weight of original fraction):							
		2.0	1.0	0.5	0.2	0.1	0.05	0.02	0.01
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	7	6	6	8	14	35	115	±
2.	H.Fb	6	5	5	8	13	34	136	±
3.	B.F.	6	5	5	6	7	10	47	115
4.	B.Fb.	7	6	5	6	8	18	126	±

* Fractions I contain about 60% coagulable fibrinogen, whereas the fibrinogens are about 90% coagulable.

2. H.Fb.: human fibrinogen, purified, courtesy of Harvard workers; 3. B.F.: bovine plasma Fraction I, courtesy of Dr. Lesh (Armour Laboratories);† 4. B.Fb.: fibrinogen, repptd. × 2 from B.F. by ¼ sat. $(NH_4)_2SO_4$; 5. Strep.: streptokinase (miscalled "streptococcal fibrinolysin") prepared according to the methods of Tillett and Garner.[13] Extraction of 100 mg with 10 cc borate buffer[1] yields a clear supernatant which is removed from the insoluble residue for use in the tests; 6. T: thrombin, enzyme-free, in the form of "rabbit hemostatic globulin," courtesy of Dr. Parfentjev (Lederle Labs).[14] The dried material is soluble in 1.0% solution in borate buffer; 7. Hep.: Sod. heparinate (Lederle), 1 mg = 100 Toronto units, 1% soln.; 8. T.I.: crystalline trypsin-inhibitor, from pancreas, courtesy of Dr. Kunitz (Rockefeller Institute, Princeton);[15] 9. S.B.I.: crystalline trypsin-inhibitor, from soybean, courtesy of Dr. Kunitz;[16] 10. Egg: commercial egg-albumen ("albumin, egg, soluble powder," Merck), a preparation in laboratory stocks for many years; 11. E.I.: a purified inhibitor from egg-white, sent us by Dr. T. E. Weichselbaum (Washington Univ., St. Louis) 6 years ago; 12. Buffer: borate buffer,[1] pH = 7.7, used as solvent and diluent throughout.

Clotting-time in relation to concentration of plasma fraction (Table I). Percentage concentration is in terms of dry weight of original material. It is not corrected for small amounts of insoluble matter and for somewhat significant contamination with non-coagulable protein. Fractions I yield about 60% true fibrin, whereas fibrinogens are about 90% clottable. The data in Table I vary slightly from experiment to experiment but the values do afford a fair approximation of the amounts of fibrinogen equivalent to the various clotting-times (c.t.) throughout the present studies. Solutions of 0.5-1.0% give optimal clotting-times and are used throughout. Such concentrations are admittedly high for maximal sensitivity of fibrinogenolytic tests but are preferred for testing their content of proteolytic enzyme system.

Fibrinogenolytic tests for tryptase in plasma fractions (H.F., H.Fb., B.F., and B.Fb.) There is no demonstrable fibrinogenolysis in these 4 plasma fractions in 24-48 hr. Clotting tests of 0.5% solutions, sampled at intervals and added to 0.5 cc T, gave c.t. of 5-6 sec. except in H.F. (48 hr.) and B.Fb. (24 hr.) where 10″ and 14″ c.t., respectively, were, due to removal of some fibrinogen by "spontaneous" clotting, of very minor importance. That minute traces of tryptase are present, nevertheless, in the human fractions is indicated by lysis of their fibrin clots on overnight incubation at 40°C. Bovine ma-

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[11] Cohn, E. J., *et al.*, *J. Am. Chem. Soc.*, 1946, **68**, 459.

[12] Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., *J. Clin. Invest.*, 1944, **23**, 557.

† Lesh prepares bovine plasma fractions on the principles worked out at Harvard.

[13] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

[14] Parfentjev, I. A., Goodline, M. A., and Clapp, F. L., *J. Lab. Clin. Med.*, 1943, **28**, 1465.

[15] Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

[16] Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

TABLE II.

Fibrinogenolysis by Varying Strength of Streptokinase Solution.

L = 5 cc H.F. + 0.5 cc buffer + 0.5 cc strep. (strengths cited), incubated at 40°C for periods stated. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%).

L.	Strength strep. %	15 sec.	¼ hr	½	¾	1	2	2½	3	4	8	18 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	0.05	6	7	8	11	13	25	35	45	55	360	∞
2.	0.1	6	9	13	21	37	174	300	∞			
3.	0.5	6	16	46	105	∞						
4.	1.0	6	19	65	186	∞						

TABLE III.

Fibrinogenolytic Tests for Tryptogen in Plasma Fractions.

L = 5 cc cited fractions + 0.5 cc strep. (1%) incubated at 40°C for periods stated. Clotting times (sec.) for 0.5 cc L + 0.5 cc T (1%).

L.	Fraction	15 sec	¼ hr	½	¾	1	1¼	1½	2	5	21 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	7	9	10	55	420	∞				
2.	B.F.	5	5	5	5	5	5	5	5	5	5
3.	H.F. B.F.	5	5	5	9	25	100	∞			
4.	H.Fb.	6	24	∞							
5.	B.Fb.	5	5	5	5	5	5	5	5	5	5
6.	B.Fb. H.F.	5	5	5	10	45	260	∞			

terials, on the other hand, are remarkably stable. B.F. remains clear and gives identical c.t., with T, for 7-10 days at room temperature. The fibrin clots show no visible change in 10-14 days at 40°C.

Fibrinogenolysis by varying strengths of streptokinase solution. (Table II). The clear supernatant from extracting 100 mg crude streptokinase with 10 cc buffer solution is decanted and filtered through glass-wool to yield a solution designated "1%," from which dilutions are made for the tests of Table II. Concentrations of 0.5-1.0% are about optimal and weaker solutions give slower fibrinogenolysis in H.F. The streptokinase extraction for use in these tests requires fresh preparations, since solutions rapidly lose potency and are inactive within 24-48 hours even at ice-box temperature. Solutions of 0.01% (or less) show no fibrinogenolysis but continue to give the same (6″) c.t. as controls (buffer only) in tests extending over 18 hours at 40°C.

Fibrinogenolytic tests for tryptogen in plasma fractions. (*Table III*). Recent workers[17-19] have shown that streptokinase is not, as long believed,[13] itself the proteolytic agent, but merely the kinase or activator of enzyme-precursor (tryptogen) in plasma. The extent to which one-tenth volume of 1% streptokinase can activate the tryptogen in the present plasma fractions is brought out by the fibrinogenolytic tests of Table III. The high content of tryptogen in H.F. and H.Fb. is clearly shown as compared with the absence of tryptase development in B.F. and B.Fb. That the failure to lyse B.F. and B.Fb. is due to lack of enzyme precursor rather than to presence of inhibitors in the bovine material is shown in Exp. 3 (and 6), where a mixture of H.F. and B.F. (or B.Fb.) shows complete lysis of *all* the fibrinogen, bovine as well as human, in very little longer time than the human alone (L_1). These data also rule out any strict species specificity and some similar experiments on dog fibrinogen add further evidence on this point.

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[17] Milstone, H., *J. Immunol.*, 1941, **42**, 109.

[18] Kaplan, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 40.

[19] Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

TABLE IV.

Fibrinogenolytic Tests of Effects of Heparin on Tryptase Formation and Stability.

Lytic mixtures at 40°C for periods stated. All reagents 0.5%. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%). L_1 (*without* heparin): tests 3-6. L_2 (*with* heparin): tests 7-10.

L.	Lytic mixture	15 sec	¼ hr	½	¾	1	2	4	6	17	24 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	10 cc H.F. + 1 cc buffer + 1 cc Strep.	6	16	46	105	∞					
2.	10 cc H.F. + 1 cc Hep. + 1 cc Strep.	25	±	∞							
3.	5 cc B.F. + 1 cc L_1 (15′ old)	6	6	6	—	6	7	11	20	480	∞
4.	5 ″ ″ + 1 ″ ″ (25′ ″)	6	6	6	—	6	6	8½	10	100	165
5.	5 ″ ″ + 1 ″ ″ (50′ ″)	6	6	6	—	6	6	7	8	35	55
6.	5 ″ ″ + 1 ″ ″ (100′ ″)	6	6	6	—	6	6	6	6	14	16
7.	5 ″ ″ + 1 ″ L_2 (15′ ″)	12	12	13	—	14	18	28	40	*180*	*340*
8.	5 ″ ″ + 1 ″ ″ (25′ ″)	12	12	12	—	13	16	20	28	140	195
9.	5 ″ ″ + 1 ″ ″ (50′ ″)	12	12	12	—	12	14	16	21	52	60
10.	5 ″ ″ + 1 ″ ″ (100′ ″)	12	12	12	—	12	13	14	17	34	38

Instability of activated tryptase and effects of heparin. (Table IV). Some preliminary tests showed that 1 cc of (H.F. + strep.) lysate had a stronger effect in lysing 5 cc of fresh H.F. if added at (or before) the time of complete lysis in the original mixture than if added ¼-½ hour later. Because of the finding that heparin apparently enhances the potency of the tryptase, and because this might possibly be due to some stabilization of the enzyme, the stability tests of Table IV were conducted with and without heparin. At incubation periods of 15, 25, 50, 100 minutes, 1 cc samples were removed from (H.F. + strep.) lytic mixtures (a) without (L_1) and (b) with (L_2) one-tenth volume of 0.5% heparin (v. reagents). On addition of the 1 cc samples to 5 cc B.F. (0.5%), and observation of the rather slow fibrinogenolysis, the activity differences are clearly brought out. The instability of tryptase occurs in both series, so there is no evidence that heparin is stabilizing the enzyme. The best explanation of the *apparent* improvement of tryptase activity in the heparinized mixtures is that there is some immediate antithrombic action of heparin.[20,21] While this merely increases the clotting-time from 6″ to 12″ while the fibrinogen is at, or close to, full strength, it would become more significant as the fibrinogen is weakened by tryptic lysis. In support of this explanation, mixtures of B.F. diluted to contain in 0.5 cc volume 0.5, 0.2, 0.1, 0.05% of protein, and all treated with 0.05 cc of heparin (diluted to give the identical concentration of the above tests) and then clotted with the usual 0.5 cc T, give clotting-times: 12″, 18″, 35″, 100″ as compared with controls (without heparin): 5½″, 6″, 7″, 10″. Obviously, the antithrombic effect of heparin is much stronger with weaker fibrinogen concentrations.

Fibrinogenolytic tests of trypsin inhibitors. (Table V). The effects of known inhibitors (of pancreatic trypsin) on the natural plasma tryptase system are illustrated by selected tests, using both H.F. and H.Fb., in the first instance, on the original protein + streptokinase mixture. Inhibition is readily revealed by the fibrinogenolytic test method and is demonstrated for (a) crystalline trypsin-inhibitors, both from pancreas (T.I.) and from soybean (S.B.I.), and (b) egg-albumen, crude (Egg.) and fractionated for trypsin-inhibitor (E.I.).

In a second approach, in confirmation of the ability of these inhibitors to inactivate fully-formed tryptase, not merely to prevent its formation (possible antikinase action), additional tests were made on mixtures: 5 cc B.F. + 0.5 cc fresh lysate (H.F. + strep. incub. 35 min.) + 0.5 cc inhibitor solution. The control (buffer only) lysed in 12 hours, at which time there was no lysis

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[20] Ferguson, J. H., and Glazko, A. J., *Am. J. Physiol.*, 1941, **134**, 47.

[21] Glazko, A. J., and Ferguson, J. H., *Am. J. Physiol.*, 1941, **134**, 54.

TABLE V.
Fibrinogenolytic Tests of Trypsin Inhibitors.
Lysis mixture (L) — 5 cc H.F. (1%) or H.Fb. (1%) — 0.5 cc strep. (1%) — 0.5 cc cited inhibitor, incubated at 40°C for periods stated. Clotting-times read at 25°C for 0.5 cc L — 0.5 cc T (1%).

L.	Fraction	Inhibitor	15 sec	1/4 hr	1/2	3/4	1	1¼	1½	2	2¼	2½	3	8	12	24 hr
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	none	5	13	27	64	95	320	∞							
2.	H.F.	egg (1%)	5	7	10	13	19	29	37	83	123	∞				
3.	H.F.	S.B.I. (1%)	6	6	6	6	6	6	6	6	6	6	6	6	6	6
4.	H.Fb.	none	6	24	∞											
5.	H.Fb.	E.I. (1%)	5	8	12	16	20	27	35	53	—	—	95	∞		
6.	H.Fb.	T.I. (1%)	6	6	6	6	6	6	6	6	—	—	6	6	6	6

(minimal 7" clotting-time) in T.I., mere trace (10" c.t.) in S.B.I. and advanced lysis (480" c.t.) in E.I. The last, therefore, acted weakly but the crystalline inhibitors were very effective. T.I. showed no lysis whatever in 48 hours, whereas S.B.I. gave clotting-times 25", 114", 168" at 24, 36 and 48 hours, respectively, showing that the tryptase action finally broke through the inhibition. It is perhaps noteworthy that tryptase activity is demonstrable after such long periods, despite the evidence (Table IV) of a definite degree of instability.

Streptokinase, etc. In the absence of plasma protease (tryptase) or its precursor (tryptogen), the streptokinase lacks proteolytic, thrombic, or thromboplastic effects. It is possible, of course, to obtain tryptase in fibrinogen-free plasma fractions. The very important thromboplastic action of this plasma protease will be reported in a subsequent communication.

Nomenclature. Similarities to pancreatic trypsin e.g. 1) alkaline pH optimum, 2) variety of proteins attacked (fibrin, fibrinogen, prothrombin, gelatine, casein, hemoglobin, etc.), 3) effects of known trypsin-inhibitors, and 4) clot-aiding actions, favor continued characterization of the plasma protease as a "tryptase" or trypsin-like enzyme. The new term *plasmin*[23] is acceptable but hardly necessary and pending isolation, perhaps somewhat premature. The ambiguous term "*fibrinolysin*," which has been used both for the tryptokinase[22] and for the active tryptase[23] should obviously be avoided.

Summary. The present study is based upon the use of a fibrinogenolytic (rather than fibrinolytic) technic for demonstration of a natural plasma protease *(tryptase)* and its precursor *(tryptogen)* in the fibrinogen-rich plasma fractions. The value of ***streptokinase*** (miscalled "streptococcal fibrinolysin") for activation of the enzyme precursor is confirmed and tests are presented to show that failure to secure proteolytic effects *e.g.* in bovine plasma Fraction I, is due to lack of tryptogen (tryptase precursor) rather than to any significant presence of tryptase-inhibitor.

Heparin, unaided, shows no enzyme-inhibitory effects in the cited tests. *Antitryptase* effects of (a) crystalline inhibitors, from pancreas and soybean, and (b) crude and fractionated egg-albumen, favor the characterization of the plasma protease as a "tryptase."

Confirmation of the existence of tryptogen, together with a trace of active tryptase, in H.F. ("antihemophilic globulin") is further, but still inconclusive, evidence that the source of the coagulation defect in hemophilia is to be sought for in the complexities of the plasma protease system.

The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University.

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[22] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

[23] Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

15772

The Serological Differentiation of Mumps Complement-Fixation Antigens.*

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The recent adaptation of mumps virus to the chick embryo by Habel[1] and others[2,3] has greatly facilitated the serological diagnosis of the disease in its various manifestations and has placed such tests within the reach of the routine laboratory. The occurrence of a hemagglutination phenomenon similar to that observed with the influenza viruses permits the determination of antibodies by the inhibition of this reaction, as shown by Levens and Enders.[2] In addition, various materials derived from the infected chick embryo have been shown to contain complement-fixation antigen. Following the inoculation of the virus by different routes antigen was found in suspensions of the allantoic,[2] amniotic[2] and yolk sacs,[1-3] and in the allantoic[1] and amniotic fluids.[1,2] Studies in this laboratory, aside from confirming the earlier observations, were concerned with the demonstration of differences in the mumps-specific antigenic properties of suspensions of infected allantoic and amniotic sacs on the one hand, and of allantoic and amniotic fluids on the other.

Methods and Materials. A strain of mumps virus adapted to the amniotic sac of the chick embryo was kindly supplied by Dr. Enders. It has been propagated in this laboratory both in the amniotic and allantoic sacs of 8-day-old chick embryos for 8 to 14 consecutive passages, using for the transfers 10- to 10,000-fold diluted amniotic or allantoic fluids, respectively. For both routes a hole with a diameter of about 1 cm was cut with scissors into the shell over the air sac after the shell had been sterilized with 70% alcohol. In the case of the allantoic inoculation the needle was slipped just under the membrane to assure injection of the seed into the allantoic sac which is still very small on the 8th day of incubation of the embryo. For the amniotic inoculation a small part of the exposed shell membrane was removed over the approximate location of the embryo and the inoculum was deposited into the amnion under full visibility of this structure. The hole in the shell was sealed with scotch tape. After further incubation of the eggs at 35 to 37°C for 5 to 7 days, the virus usually attained maximal concentration in the respective embryonic fluids as measured by infectivity titrations in the chick embryo, hemagglutination and complement-fixation tests. At this time the allantoic and amniotic fluids and the corresponding membranes were harvested separately. The membranes were emulsified by means of a Waring blendor in saline solution to form a 10 or 20% suspension.

Infectivity titrations were performed in 8-day-old chick embryos by the injection of the virus preparation diluted in steps of 10 in broth by the allantoic (0.5 ml inoculum) or the amniotic routes (0.2 ml inoculum) depending on the passage series of virus used.

For the hemagglutination test 0.4 ml of virus suspension was mixed with 0.2 ml of a 1% suspension of washed fresh chicken red cells and the mixtures were incubated at 4°C until the erythrocytes had settled. The degree of agglutination was determined by the pattern formed by the cells at the bottom of the test tubes.

The complement-fixation technic was the same as employed for the studies on the antigenic differentiation of influenza antigens.[4] Dilutions of antigen, serum and

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* The work described in this paper was aided by the Office of Naval Research.

[1] Habel, K., *Pub. Health Rep.*, 1945, **60**, 201.

[2] Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

[3] Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 15.

[4] Wiener, M., Henle, W., and Henle, G., *J. Exp. Med.*, 1946, **83**, 259.

guinea pig complement were mixed using 0.1 ml amounts of each. The mixtures were incubated at 37°C for one hour before the addition of 0.2 ml of sensitized sheep red cells (2.5% suspension). After further incubation for 20 minutes at 37°C and overnight at 4°C, the tests were read by estimating the degree of hemolysis obtained. Two units of amboceptor and 1.3 units of "Lyovac" complement† were employed. The highest initial dilutions of antigen or serum giving complete fixation of complement (no hemolysis) were recorded as titers. Most tests were performed according to optimal titration technic since zone phenomena were observed frequently in agreement with earlier observations by Enders and co-workers.[5] Human convalescent sera were used in these studies and the results were compared whenever possible with those obtained with sera from the same patients taken during the first days of the disease. Control antigens were prepared from suspensions of allantoic and amniotic membranes derived from normal embryos or from eggs infected with influenza B virus, in a manner corresponding to that used for the mumps preparations.

Experimental. Upon allantoic inoculation of mumps virus of the allantoic-passage series, complement-fixation antigen was found in the allantoic fluids and membranes and in the amniotic sacs, but none or little in the amniotic fluids. The allantoic tissue usually contained higher titers of antigen than the amniotic sac. Conversely, following amniotic injection of virus of the amniotic-passage series, higher titers of antigen were noted in the amniotic sac than in the allantoic tissue and, in early passages, the allantoic fluid gave negative or weak reactions in contrast to the strongly positive results with the amniotic fluids. Normal allantoic and amniotic fluids did not react with any of the sera used, whereas positive reactions with the suspensions of normal allantoic and amniotic membranes were obtained with some of the sera in low dilution. The antigen titers in the infected fluids, varying between 1:2 and 1:8, were lower than those observed in the corresponding membranes which were found to lie between 1:16 and 1:128. Hemagglutination tests, on the other hand, showed high titers in the fluids (1:256 to 1:1024 for allantoic, and 1:1024 to 1:8192 for amniotic fluids) but were low or negative with the suspensions of membrane. Although interpretation of the results of hemagglutination tests with the tissue suspensions has to take into consideration the inhibitory effect of some component in the emulsion, the data are comparable to those of infectivity titrations. In these the fluids revealed titers 100 to 1000 times higher than the suspensions of the membranes. The number of 50%-infectivity doses (ID_{50}) in the fluids varied from $10^{7.3}$ to $10^{8.8}$ per ml in the reported experiments, whereas the suspensions of membranes gave values between $10^{4.5}$ and $10^{5.8}$. These observations failed to show a correlation between the infectivity and hemagglutinin titers on the one hand, and the concentration of complement-fixation antigen on the other. This discrepancy has already been noted by Levens and Enders.[2]

These data suggested further comparisons among the various materials in regard to the nature of the antigens they contained. By the use of high speed centrifugation it could be shown that the mumps antigens of allantoic and amniotic fluids were sedimented together with the virus particle at 20,000 r.p.m. for 20 minutes. The infective, hemagglutinating and complement-fixing properties were found in the sediment whereas the supernatant fluid gave negative complement-fixation tests and only low titers of virus and hemagglutinin. This is in agreement with the findings of Beveridge and Lind.[6] From the tissue suspensions, on the other hand, only part of the complement-fixation antigen was removed by this procedure although the infectivity of the supernatant fluid showed a decrease in titer comparable to that observed with infected allantoic and amniotic fluids. The hemagglutination re-

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† Generously supplied by Sharp and Dohme, Inc.

[5] Enders, J. F., Cohen, S., and Kane, W. W., *J. Exp. Med.*, 1945, **81**, 119.

[6] Beveridge, W. I. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 127.

TABLE I.
Differential Centrifugation of Allantoic Fluid and Membrane Suspension Infected with Mumps Virus.

	Allantoic fluid				Allantoic membrane			
	Infectivity (allantoic inoculation), ID_{50}/ml	Hemagglutination titer	Complement-fixation maximal titer		Infectivity (allantoic inoculation), ID_{50}/ml	Hemagglutination titer	Complement-fixation maximal titer	
Preparation			Antigen	Serum			Antigen	Serum
Original	$10^{7.5}$	1:1536*	1:8†	1:32†	$10^{5.5}$	0	1:64	1:32
20,000 r.p.m. supernate	$10^{5.3}$	1:8	0		$10^{2.6}$	0	1:32	1:32
20,000 r.p.m. sediment 2 × conc.	$10^{8.3}$	1:4096	1:16	1:64	$10^{5.3}$	1:32	1:16	1:64
30,000 r.p.m. supernate	$10^{3.1}$	0	0		$10^{2.8}$	0	1:4	1:32
30,000 r.p.m. sediment 2 × conc.	$10^{3.3}$	0	0		$10^{1.8}$	0	1:16	1:32

* Initial dilution of the preparation giving last definite agglutination of red cells.
† Initial dilution of serum or antigen giving last complete fixation of complement.
All serum and antigen controls, as well as controls with acute serum and with normal membrane preparation gave negative results.

action, which usually was negative in the original suspensions, became distinctly positive in the resuspended sediments. This finding may be explained by the assumption that the hemagglutinins have been separated by the centrifugation procedure from an inhibitor present in the original suspension.‡ Recentrifugation of the 20,000 r.p.m.-supernate at 30,000 r.p.m. for one hour removed a large part of the "soluble" antigen, but some of it still remained in suspension. An experiment of this type, employing infected allantoic fluids and the corresponding membranes as the source of antigen, is shown in Table I. As pointed out, control antigens prepared from normal allantoic and amniotic sacs gave positive reactions with a few of the sera in low dilution. The material showing this reactivity was in part sedimented at 20,000 r.p.m., the supernatant being usually inert. It is obvious then that tissue antigens for diagnostic tests should be subjected to high speed centrifugation in order to avoid some of the nonspecific reactions.

The centrifugation experiments showed that physical differences existed between the antigen present in the embryonic fluids and at least part of the antigen present in suspensions of the membranes. In further experiments comparisons were made between preparations of infected allantoic fluid on the one hand, and the supernatant fluid after centrifugation at 20,000 r.p.m. of suspended allantoic membrane on the other; the former representing mostly large antigen particles which are sedimentable with the virus activity, the latter containing a smaller antigen not directly linked with the infective property. These comparisons showed on occasion differences in the optimal titration patterns of the 2 antigens but not as frequently and as markedly as was noted in the case of the corresponding influenza antigens.[4,7] More convincing evidence of serological differentiation was obtained by absorption of convalescent sera with the 2 types of

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‡ Similar results were obtained upon dialysis of suspensions of infected membranes.

[7] Henle, W., and Wiener, M., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 176.

TABLE II.
Results of Serum Absorption Tests.

Serum absorption				Optimal complement fixation reaction Antigen							
				Allantoic fluid		Allantoic membrane					
							20,000 R.P.M.		30,000 R.P.M.		
Material	ID_{50}/ml serum	Units of antigen/ml serum	Dilution of serum	Orig. und.	20,000 R.P.M. sediment und.	Orig. 1:16	Supernate 1:16	Sediment 1:2	Supernate 1:4	Sediment 1:8	Saline
—	—	—	1:8	0	0	0	0	0	0	0	c
			1:16	0	0	0	0	0	0	0	c
			1:32	0	0	0	0	0	0	0	c
			1:64	tr	tr	wk	wk	0	st	tr	c
Allantoic fluid 20,000 R.P.M. sediment	$10^{9.4}$	320	1:8	st	st	0	0	0	0	0	c
			1:16	c	c	0	0	0	wk	0	c
			1:32	c	c	ac	ac	ac	c	ac	c
			1:64	c	c	c	c	c	c	c	c
Allantoic membrane 20,000 R.P.M. sediment	$10^{5.9}$	1280	1:8	0	0	c	c	c	c	c	c
			1:16	0	0	c	c	c	c	c	c
			1:32	ac	tr	c	c	c	c	c	c
			1:64	c	c	c	c	c	c	c	c
Allantoic membrane 30,000 R.P.M. sediment	$10^{2.2}$	320	1:8	0	0	c	c	st	c	c	c
			1:16	0	0	c	c	c	c	c	c
			1:32	0	0	c	c	c	c	c	c
			1:64	ac	tr	c	c	c	c	c	c

0—no hemolysis; tr—trace; wk—weak; st—strong; ac—almost complete; c—complete hemolysis.

TABLE III.
Antibody Content of Various Selected Human Sera.

Case No.	Age (years)	History	Time after onset of disease	Serum antibody titer against		
				Virus-bound antigen (allantoic fluid)	Soluble antigen (Supernate 20,000 R.P.M. of allantoic membrane)	Normal allantoic membrane
1	4½	mumps	3 days	1:4	1:16	1:2
			15 ”	1:64	1:64	1:2
			29 ”	1:128	1:128	1:2
			46 ”	1:128	1:64	1:2
2	5½	”	7 ”	1:16	1:16	0
3	4	”	7 ”	1:16	1:32	0
			17 ”	1:64	1:64	0
4	6½	”	4 ”	1:16	1:8	0
			14 ”	1:64	1:32	0
6	4	”	4 ”	1:4	1:8	<1:4*
			16 ”	1:32	1:64	<1:4
36	9	”	2 months	1:64	1:8	0
35	7	”	3 ”	1:32	1:8	0
31	8	”	2 years	1:8	0	0
28	2	”	2 ”	1:8	0	0
11	4	”	2 ”	1:4	0	0
25	10	”	2 ”	1:8	0	0
13	11	”	2 ”	1:4	0	0
44	9	unknown	—	1:16	0	0
43	6	”	—	1:8	0	0
42	9	”	—	1:8	0	0
19	8	inapparent mumps	16 days†	1:128	1:32	0
16	6	” ”	39 ” †	1:16	1:4	0

* Partial fixation of complement in serum dilution 1:4.

† Negative serum obtained 16 and 39 days, respectively, prior to the recorded specimen.

antigen. Human convalescent serum was absorbed with the sediments obtained by centrifugation at 20,000 r.p.m. from infected allantoic fluid or suspension of allantoic membrane, or with the material sedimented at 30,000 r.p.m. from the suspension of membrane after removal of the larger particles at 20,000 r.p.m. The ID_{50} for chick embryos as well as the units of antigen used for the absorption per 1 ml of serum were determined and are recorded in Table II. When mumps convalescent serum was absorbed with the antigen sedimented at 20,000 r.p.m. from allantoic fluid (virus particles) it no longer reacted with this antigen nor with the original allantoic fluid. It gave, however, distinctly positive reactions with suspensions of infected membrane and the fractions derived therefrom, although a decrease in serum titer was noted. On the other hand, absorption of the serum with the smaller antigen sedimented at 30,000 r.p.m. from suspensions of allantoic membrane previously cleared by centrifugation at 20,000 r.p.m. removed the reactivity with the "soluble antigen" but left antibodies to the allantoic fluid antigen and the sediment derived therefrom. Absorption of the serum with the particles sedimented from the membrane suspension at 20,000 r.p.m. gave results corresponding to the effect of absorption with the soluble antigen rather than with the virus particles obtained from allantoic fluid. The reasons for this latter finding are not clear as yet. The infectivity titrations of the suspensions of membrane used for the absorption showed only 0.1% of the activity of the allantoic fluid material. Consequently, there might not have been a sufficient concentration of virus to remove the antibodies to the virus-bound antigen. On the other hand, antibodies against the soluble antigen were completely removed by the particles sedimented from the membranes at 20,000 r.p.m. It is possible then that the "soluble antigen" occurs in different physical states, *i.e.*, in different particle sizes either *per se*, or as a result of adsorption onto particulate components of the host cell. One also might consider the possibility that the virus may occur in a different phase in the tissues, *i.e.*, covered with the soluble antigen, as compared to the "naked" virus in the allantoic fluid. This problem requires further investigation. Control absorptions of the convalescent serum with fractions sedimentable at 20,000 and at 30,000 r.p.m. from normal allantoic membranes or from tissues infected with the Lee strain of influenza B virus did not affect the reactivity of the serum with either the mumps virus as contained in the allantoic fluid or the mumps soluble antigen present in the suspension of membrane.

Further evidence of the serological differentiation between the 2 antigens was obtained in the study of the reactivities of various human sera with these preparations. Marked titers in antibodies against both antigens were found in sera of children convalescent from mumps and those having undergone a recent inapparent infection. Some sera from individuals with a history of long past infection or from subjects with unknown exposure showed antibodies only against the virus-bound antigen but not against the soluble material. Some of these cases are listed in Table III. This finding emphasizes again the difference between the 2 antigens; the one dominantly present in the embryonic fluids, the other in the suspensions of infected tissues. These results suggest, in addition, that the antibodies against the virus-bound antigen persist for a longer period of time following the infection than antibodies against the soluble substance. This, however, can be verified only by following the concentration of the 2 antibodies in individual patients over a prolonged period of time. In a few cases it was noted, too, that the antibodies to the soluble material may become measurable and reach higher concentrations earlier than the antibodies to the virus-bound antigen.

The demonstration of 2 distinct complement-fixing antibodies, and particularly the fact that one of them, the antibody against the soluble substance, may be absent from certain sera tends to explain discrepancies in the correlation of the results of complement-fixation tests with the susceptibility of an individual. If only tissue extracts are employed as antigen which contain mainly the soluble material a negative test may be recorded in a subject with a history of mumps at some time in the past. The use of the virus-bound antigen may properly classify such individuals. This latter antigen will also demonstrate past contact with the virus even though the history may not reveal past apparent infection.

The relation of the antibodies against the virus-bound antigen to the neutralizing and hemagglutination-inhibiting antibodies is under investigation at present. A comparison of the results of complement-fixation tests with these 2 antigens and skin tests is indicated.

Summary. Study of the complement-fixation antigens obtained from chick embryos infected with mumps virus revealed 2 antigenically distinct components separable by high speed centrifugation. One antigen is closely linked with the virus and found predominantly in the allantoic and amniotic fluids; the other component is smaller and present mainly in the allantoic and amniotic membranes. The 2 antigens can be differentiated by serum absorption technic. Some human sera contain antibodies to the 2 antigens (early convalescent sera) whereas others may show antibodies only to the virus-bound antigen but not to the soluble material (long past infection).

15773 P

The Localization of Radiophosphate in Cells.

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The exact mode in which ions are distributed in protoplasm is at present unknown. Using radioactive isotopes, several authors have attempted, by placing photographic film in close contact with tissues and thus obtaining a radio-autograph, to record where added ions are localized in the cell. Unfortunately this procedure has not yielded any great amount of information on cellular localization, although excellent differentiation of different tissue masses has been obtained.[1] The difficulty in obtaining clear radio-autographs of cells is that the cell diameters are of the same order of magnitude as the distance that β-rays, not normal to the film, travel before striking the film. The net result is a general fog which obscures the desired detail. To obtain pictures with more detail it is necessary therefore either to collimate or to focus the electrons coming from the tissue to the film, or else to use cells of much larger dimensions. In this study the second alternative has been employed in that cells of *Nitella* or *Chara* sp., which have diameters of the order of 1-2 mm and lengths of about 40 mm, were used.

Experimental. Cells of *Chara* or *Nitella* were prepared for use as previously described.[2] The cells were removed from pond water, blotted to remove excess water and placed on end in a tube containing 0.001M radioactive sodium phosphate solution at 15°C and pH 7. In some experiments only enough phosphate solution was used to cover 1-2 mm of the cell as measured along its axis. The tube was then filled with washed paraffin oil. By this arrangement it was possible to expose only a minimal part of the surface of the cell to phosphate solution and hence obtain some idea of the rapidity of diffusion and possible circulation of the phosphate due to cyclosis. To be sure there must be a thin film of water extending up from the PO_4 solution along the cell surface but this method probably lets ion diffusion proceed as asymmetrically as possible. After various times of immersion the cells were removed from the tubes, washed thoroughly in running distilled water, placed on a glass slide and rapidly frozen and dried ***in vacuo***. A piece of X-ray film was then placed over the dried cell and exposed for a suitable time.

Results. Some photographs resulting from these experiments are shown in Fig. 1. In photo "A" the cell *(Nitella)* was immersed in radioactive phosphate solution (P*) from its end marked by an arrow (↓) for a distance of 25 mm along its axis for a period of 25 minutes. As is to be expected, the P* concentration is highest in this region, but it can also be seen that the P* concentration at the node of the cell is quite high. This may be due to the greater mass of protoplasm here or to the more rapid metabolic turnover in this region, or to a combination of both. In photo "C" a cell which was immersed in P* solution for 25 min. to a depth of only 2 mm is shown. Directly below the radio-autograph (black background) is shown a photo of the cell taken by transmitted light. (The cell was fractured in placing photographic film upon it). It can be seen from this photo that although there is a slight amount of activity in the nodal region, the P* is mainly concentrated in rather discrete zones along the cell. This cell was *Chara*. The *Chara* cell generally has a rather heavy incrustation of salt deposits on its wall. It is possible that these localized zones of P* concentration represent the

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[2] Brooks, S. C., *Trans. Faraday Soc.*, 1937 **33**, 1002; Proc. Soc. Exp. Biol. and Med., 1938, **38**, 856.

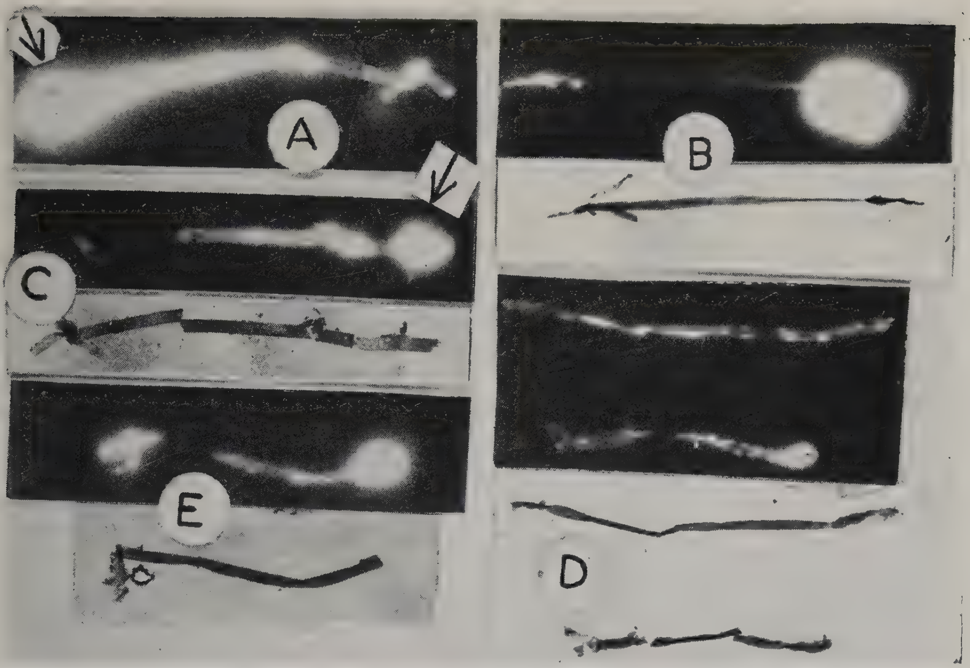

FIG. 1.
Showing the distribution of radiophosphate in *Nitella* and *Chara*. (See text.)

exchange of P* into such crystals, although the zones of concentration do not correspond with the visible areas of incrustation. (See transmitted light photo).

In photos "E" and "B" are shown radio-autographs taken after 60 and 90 minutes of immersion of cells to a depth of 2 mm. Both show concentration of P* in the node, and some slight localization of P* along the axis. Both cells are *Nitella*. Photo "D" is a radio-autograph of 2 *Nitella* cells together with transmitted light photographs taken after 660 min. immersion in P* solution to a depth of 2 mm as measured along the cell axis from the arrow. Here the P* has been distributed throughout the cell, but in discrete patches. One possible interpretation of such distribution is that in the course of vacuum drying of the cells, the protoplasm aggregated in clumps. This does not seem likely inasmuch as the transmitted light photos show that the chloroplasts must be rather evenly distributed throughout the cell as it is rather uniformly opaque. Microscopic observation confirms such a view. There is also the possibility that in the drying of the cell P* which has diffused throughout either or both cell wall and protoplasm crystallizes in quite large crystals. The distribution of P* in these photos, however, seems too sparse to render such a view likely. It would seem that P* in these algal cells is found in areas where there was already a high concentration of inactive PO_4 for exchange, and that the pictures obtained in this study represent the way P* is distributed as it diffuses along the cell.

My thanks are due to Professor S. C. Brooks for the use of his laboratory and for many helpful suggestions.

15774

Studies on the Pathogenesis of Brucellosis.*

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The mechanism by which Brucella produces infection and continues to grow in the tissues of immune individuals has been suspected but not clearly demonstrated. One of the most interesting features of the disease in man is the prolonged sequence of periods of illness and apparent recovery for which it has been given the name of undulant fever. The finding of Brucella for months and even years after the beginning of the infection places this disease in a class with tuberculosis and syphilis, in which the infecting organism continues to multiply in spite of the considerable degree of immunity displayed by the host. However, brucellae are not quite resistant to cellular and humoral defences as is the case in tuberculosis. Therefore, its ability to remain active within the organs and eventually to be found in the blood stream must depend on factors peculiar to the microorganism.

One of the most interesting observations on the relationship of Brucella to the infected tissues was made early in the history of the disease, when Theobald Smith demonstrated that a considerable number of *Brucella abortus* crowded the cytoplasm of the cells of the chorium in the placenta of aborting animals. This finding did not seem to have been fully appreciated as a possible guide in the explanation of the remarkable persistence of Brucella in the organism in spite of the coexistence of high degree of immunity.

Corroborating Smith's findings, Goodpasture and co-workers[1] in a study of bacterial infection in the developing chick embryo found that Brucella multiplied within the cytoplasm of certain cells more intensely than could be observed in extracellular positions. More recently Buddingh and Womack[2] following the method of Goodpasture found that *Br. abortus* and *suis* had a tendency to select cells of mesodermal origin while the melitensis variety was only found in ectodermal cells. The 3 brucellae could be found in considerable numbers within macrophages not merely phagocytized but actually multiplying within the cytoplasm of such cells. These authors advanced the idea that the monocytes acting as a natural means of defense against the infecting agent supplied a favorable refuge where multiplication took place and therefore, constituted the source of continual infection from cell to cell. The intracellular position of Brucella in tissues of infected persons was first reported by Meyer[3] in a case of acute infection with *Br. suis*. The brucellae were easily seen within parenchyma cells of the kidney. After Meyer's report we decided to investigate intracellular brucellae in fatal cases of human brucellosis. It was discouraging to find so few visible organisms while cultures from the organs showed considerable numbers of colonies. Therefore, it was considered of interest to become familiar with the microscopic aspect of the disease in experimental animals. The following is a presentation of some findings after inoculation of guinea pigs, rabbits and mice with the 3 varieties of Brucella. Guinea pigs and rabbits were inoculated intravenously and also directly into the testicle. Mice were inoculated by intravenous and intranasal routes. The strains used as inoculum were *Br. abortus* and *suis* sent to us by Dr. Huddleson and a recently isolated

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* Read at the First Interamerican Brucellosis Meeting, Mexico, D.F., October 28, 1946.

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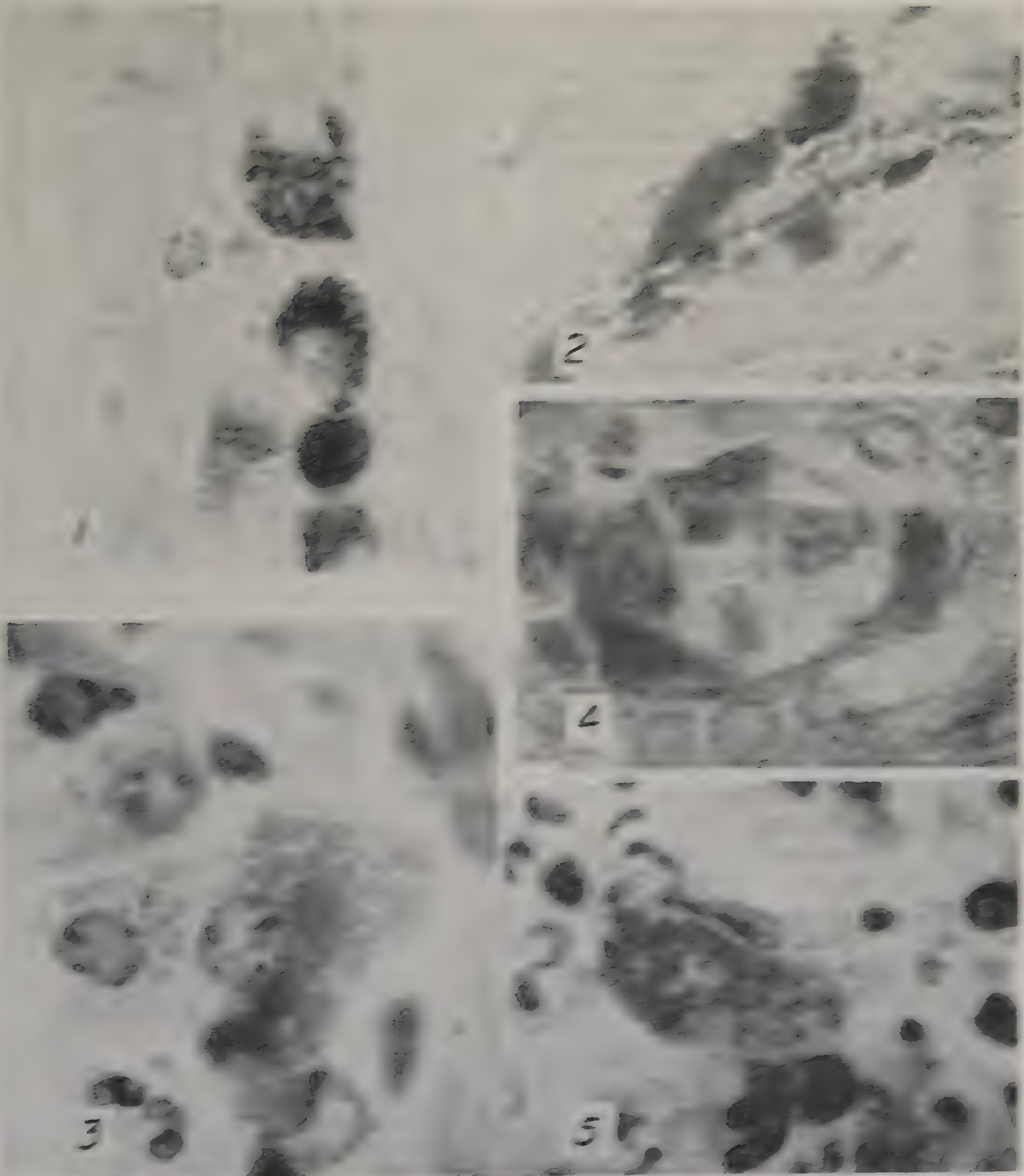
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prints made from the organs were stained with our methylene-blue-safranin stain for rickettsiae. The sections were stained with formalinized Giemsa or by Nyka's stain.[4] The latter producing delicately stained cellular elements in which violet stained brucellae could be easily found.

It is unnecessary to include here the observations concerning the cytological response to the infecting organism which will be reported by one of our associates.

The search of brucellae in smears or prints from animals inoculated intracardially showed relatively few extracellular organisms in the spleen and liver, and even less in other organs. Often one could detect polymorphonuclear leucocytes containing few phagocytized organisms and also macrophages containing brucellae in the cytoplasm. The optimum time for the finding of parasitized macrophages was about the 5th day after inoculation. Some of the mononuclear cells contained rather large numbers of brucellae but the microscopic aspect of the parasitized cells was far from being suggestive of intracellular multiplication as reported by Buddingh and Womack. On the other hand, the observation of smears from the testicle of animals inoculated locally showed cells with the cytoplasm crowded with brucellae. In smears from the locally infected testicle there were found a considerable number of polymorphonuclear leucocytes phagocytizing brucellae and also many macrophages stuffed with organisms. Although in sections of the liver and spleen it was not difficult to find infected cells, the testicle afforded better material for the study. Fig. 1 shows cells from the testicle of a guinea pig infected with *Br. melitensis* which leave little doubt of the intracellular growth in cells which are likely to be macrophages. Fig. 2 shows the growth of *Br. abortus* in the cytoplasm of a fibroblast and in Fig. 3 there is no doubt about the multiplication of *Br. abortus* within a cell, probably an interstitial cell, if compared with neighboring cells of similar morphology. In Fig. 4 it seems that cells from a capillary wall have been parasitized by *Br. abortus*. Most of these preparations were made from animals killed from the 3rd to the 5th day, and it has been our impression that the parasites within the cells have increased not because of phagocytosis but by the actual development of Brucella colonies after some of the organisms gained entrance into the cytoplasm. It is interesting to find so few or even no extracellular organisms close to the parasitized cells. However, in certain portions of the testicle one could see large numbers of extracellular organisms and it was usually in zones in which considerable numbers of polymorphonuclear leucocytes were present.

We have not yet sufficient information concerning the type of cells which allow brucellae to develop in the cytoplasm, nor the possible selectivity of the 3 varieties of organisms for a special group of cells. We can only indicate that we found macrophages, fibroblasts and, particularly in the spleen, endothelial and reticular cells which contained brucellae in the cytoplasm. To this we may add parenchymatous cells from the kidney and interstitial cells of the testis.

The inoculation of mice by intravenous or intranasal route has afforded a practical method for the study of the relationship of brucellae to the cells of the infected animal. Even 48 hours after the inoculation it has been possible to find parasitized cells in various organs. In the lung the alveolar cells are found to be parasitized in a similar manner to that which has been observed in typhus. Fig. 5 shows the aspect of an infected alveolar cell which does not look unlike similar cells parasitized with typhus rickettsiae. We have also seen macrophages stuffed with brucellae and polymorphonuclear leucocytes containing phagocytized organisms, usually in small numbers. It is interesting to find that, contrary to what has been observed in typhus-infected animals the bronchial epithelium seems to have no tendency to favor multiplication of brucellae.

Comment. There seems no doubt that most workers experienced in the clinical aspect of brucellosis consider that some of the symptoms shown by the patients are due to a state of hypersensitivity to brucellar antigens. Since 1938 we have advocated this view based on the observation of typical manifestations of allergy and the favorable effect of desensitization of the patients. To illustrate our discussion we shall give a brief description of the general aspect of the caprine type of brucellosis. The disease most frequently begins like typhoid or typhus infection or less frequently as a mild febrile influenza-like infection. The first attack, of variable duration, represents the cyclical phase of the infection ending when the individual has developed sufficient immunity to prevent further injury by the infecting agent. Then, a state of convalescence closes the incident. But in a great number of cases Brucella continues to grow as indicated. The cell to cell infection as suggested by Buddingh and Womack insures the preservation of the organism in spite of the state of immunity of the patient. The constant discharge of brucellar products into the system increases this state of immunity and at a certain moment the reactivity of the tissues and the general reactions acquire sufficient intensity to produce considerable discomfort and fever. This constant irritation may become very harmful, overcome the body defenses and even allow considerable multiplication of brucellae either focally or again as a general infection. An important factor in the failure to destroy Brucella in the immune individual seems to be the reduction in numbers of the polymorphonuclear leucocytes.

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[4] Nyka, W., *J. Pathology and Bacteriology*, 1945, **57**, 317.

The demonstration of brucellae within cells of various types is a clear indication of the mechanism of preservation of the organisms in the immune individual but most important, for the understanding of the pathogenesis of the numerous clinical manifestations of the infection, seems to be the facility with which Brucella are phagocytized by macrophages which may be transported to all parts of the body and become the source of new colonies since the cytoplasm supplies suitable material for the multiplication of the organisms.

Up to the present time we have studied relatively few animals from which only a limited number of preparations have been suitable for the investigation of intracellular brucellae, therefore, we have not been able to gather sufficient information concerning special affinity of the 3 varieties of the organism for certain cells as observed in the chick embryo by Buddingh and Womack.

According to the observations made by various authors, in experimental brucellosis and in human material, the histological lesions are, fundamentally, the nodules described by Fabyan. The nodular formation could be explained as a reaction around wandering macrophages carrying brucellae or local parasitized cells, which are surrounded first by monocytes and perhaps polymorphonuclear leucocytes and walled off by lymphocytes. It is obvious that in brucellosis there is a considerable call for the latter cells and that the infected individual endeavors to supply them. It seems likely that this mechanism of focal growth of Brucella followed by the process of nodule formation explains many of the complications of brucellosis including nervous manifestations, abscesses, focal necrosis, etc. The tendency of brucellae to select the spleen as a refuge during the course of the disease is probably due to exceedingly rich material suitable for the intracellular growth and the hyperplasia observed in this organ must be accounted for the manifestations of hypersplenism often observed in the patients.

Conclusions. The studies referred to, and our own observations in experimental brucellosis are in our opinion, good evidence of the ability of Brucella to use the cytoplasm of a variety of cells as a source of material for its growth. The fact that even in the early stages of the infection, when the host has not developed immunity against brucellae, important groups of this organism are found only within the cytoplasm of certain cells, seems to indicate that the intracellular growth is the most convenient for the preservation of the infecting agent. When the intracellular colony has reached its maximum, the cells are destroyed and the brucellae exposed to unfavorable conditions, namely to phagocytosis by polymorphonuclear leucocytes. From the scarcity of extracellular organisms one may think that the intracellular fluids are not suitable for an active multiplication of Brucella, exception is made of zones where the tissues have been subject to considerable damage. In these zones one may find extracellular Brucella in large numbers. One would consider that the organism could be placed together with true intracellular parasites, but this is not the case since Brucella have its own enzyme systems with which it is able to use rather ordinary material for its metabolism.

Considering as likely that in man Brucella grow in a manner similar to that which has been observed in the experimental infection, the pathogenesis of the disease seems to us better understood.

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Thromboplastic Action of Plasma Protease (*Tryptase*).*

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Indirect evidence, based on experimental analogy with trypsin, papain, etc. was reviewed[1] in support of the theory that blood coagulation normally involves natural plasma protease *(tryptase*[1] or *plasmin*[2] or *fibrinolytic enzyme*[3]*)* apparently identical with the agent responsible for fibrinogenolysis, fibrinolysis, and other proteolytic phenomena. Plasma tryptase resembles pancreatic trypsin in many respects, especially relevant to these problems, but does differ in 1. origin,[4] 2. kinase-specificity,[3] and 3. other important ways.[2] The following experiments confirm some similarities and lead to *direct* evidence that plasma tryptase can participate as a "thromboplastic" factor in the blood-clotting system.

Reagents. In the first paper[5] of this series, on the activation and inhibition of the fibrin(ogen)olytic protease (tryptase) in certain plasma fractions, are described: 1. Borate buffer, pH = 7.7, *viz.* 45 vol. 2.5% H_3BO_3, 45 vol. 0.5% NaCl, 10 vol. 4% $Na_3B_4O_7$, $12H_2O$, used as solvent and diluent (to constant volume) throughout; 2. Harvard (courtesy Drs. E. J. Cohn, J. T. Edsall, and colleagues) human plasma Fraction-I (H.F.), which contains about 60% fibrinogen, a trace of active protease (tryptase) and considerable enzyme-precursor (tryptogen) that can be activated by a suitable kinase (Table I, A); 3. Armour's (courtesy Dr. J. B. Lesh) enzyme-free[5] bovine plasma Fraction-I (B.F.), of about the same fibrinogen content as H.F.; 4. Thrombin (THR.), a 1% soln. of lyophilized rabbit "hemostatic globulin," Lederle's (courtesy Dr. I. A. Parfentjev); 5. Streptokinase (STREP.), the streptococcal material (Garner and Tillett, 1934) of which a 1% extract is an excellent kinase-type activator of the plasma protease; 6. Crystalline trypsin-inhibitors, protein (or polypeptide) in nature, from pancreas (T.I.)

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[*] This report is the second of a series of investigations on "Enzymes and enzyme-inhibitors, in relation to blood coagulation and hemorrhagic diseases," aided by a grant from the John and Mary R. Markle Foundation.

The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University.

[1] Ferguson, J. H., *Science*, 1943, **97**, 319.

[2] Christensen, L. R., and Macleod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

[3] Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331; 337, *et seq.*

[4] Tagnon, H. J., *Arch. internat. physiol.*, 1942, **51**, 472.

[5] Ferguson, J. H., Travis, B. L., and Gerheim, E. B., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 285.

TABLE I.

Thromboplastic Action of Plasma Protease (Tryptase) Compared with Pancreatic Trypsin.

A. *Preparation of Activators. Timing of Fibrinogenolysis.* Clotting-time test at 24°C.

	Mixture (cc)	¼ min.	¼	½	¾	1	1½ hr (incub. at 39°C for lysis)
		sec.	sec.	sec.	sec.		
A.	4.0 H.F. 0.4 STREP.	6	15	45	85	+	∞
B.	4.0 H.F. 0.4 TRYP. (20 unit)	6	21	51	90	+	∞
C.	4.0 H.F. 0.4 buffer	heat-defibrination at 45°C for 3 min.; filtered through glass wool.					

B. *Prothrombin Activation Tests.* Recalcified PRO.-A.

Clotting-times (c.t.), in seconds, at 24°C, pH = 7.7 (borate buffer) for 0.5 cc B. F. + 0.25 cc T, sampled at stated incubation periods (i.t.).

T.	Activator	¼ min.	½	1	2	6	18	24 hr	4 day	7 day	(i.t.)
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	
1.	None	268	228	195	163	110	72	55	28	13	(c.t.)
2.	Mixture C	307	247	207	172	98	68	55	28	12	
3.	,, A	324	132	86	62	45	36	28	20	12	
4.	,, B	335	58	40	27	13	8½	7	5	5	
5.	40-unit TRYP.	—	5	5	5	6	15	19	100	—	

and soybean (S.B.I.), 0.2%, courtesy Dr. M. Kunitz (Rockefeller Institute, Princeton). The present studies also use 7. a Harvard (courtesy Dr. J. T. Edsall) human plasma protein fraction (III-3), 0.1%, possessing proteolytic and thrombic properties; 8. Purified prothrombin (PRO.), 0.2%, from lyophile-dried bovine preparations supplied through the courtesy of Dr. W. H. Seegers[6] (Wayne Univ., Detroit): PRO.-A being stated to yield 15,200 thrombin units per mg tyrosine N, while PRO.-B is somewhat less purified (see below); 9. Pancreatic trypsin (TRYP.), from 2% stock solution (in glycerol-borate) prepared from commercial (Fairchild Bros. and Foster) trypsin, and diluted to 40 or 20 "unit" strength (1 cc ≡ 1 mg = 100 "units," by fibrinogenolytic test);[7] 10. Rabbit brain thromboplastin (tpln.), 0.5%, from a commercial (Difco) preparation, devoid of significant proteolytic activity; 11. M/10 $CaCl_2$(Ca).

Prothrombin. Data on the properties, especially activation and stability, of Seegers' purified prothrombin preparations will be reported in detail elsewhere, but it is relevant to note: 1. some active thrombin is present and increases on standing at room temperature in borate buffer solutions (Table II, 6); 2. there is a very slow (increased) activation to thrombin on adding calcium salt (Table I, B, 1; Table II, 1), which, in comparison with the prompt and complete activation by Ca + tpln. (Table II, 2), shows either an ultimate reaching of the same optimum in 2-7 days (PRO.-B) or incomplete activation even after a week (PRO.-A), although still capable of reaching the optimum on the subsequent addition of thromboplastin. The obvious suggestion is that the prothrombins are contaminated with small but significant traces of thromboplastic factor (? phospholipid): 3. activation by 1/10 vol. of 40-unit trypsin (and Ca) to optimal thrombic activity in about ½ hr., followed by weak thrombinolysis (Table I, B, 5).

Fibrinogenolysis test. When the natural tryptase in H.F. (human plasma fraction-I) is activated by streptokinase and the fibrinogenolysis[5] followed by the lengthening clotting-times of 0.5 cc samples added to 0.5 cc thrombin (THR.), after successive incubation periods of the lytic mixture, as illustrated in Table I, A, mixture A, the data show close similarity to lysates of H.F. and weak pancreatic trypsin (1/10 vol. of 15-20

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[6] Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

[7] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 243.

TABLE II.
Inhibition of Tryptase-Activation of Prothrombin.

5 cc vol. of thrombic mixtures (T), containing 2 cc PRO.-B + 0.25 cc Ca and activators ± inhibitors noted, incubated at 24°C, pH = 7.7, for periods stated (i.t.). Clotting-times (sec.): 0.25 cc T + 0.5 cc B.F.

T.	Activator	Inhibitor	¼ min.	¼	½	1	2	6	24 hr	7 day (i.t.)
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	(Ca only)	0	218	167	133	87	45	30	13	4 (c.t.)
2.	tpln. (0.25 cc)	0	78	4	4	4	4	4	4	4
3.	III-3 (2.0 '')	0	77	20	14	10	10	9	8	5
4.	III-3 (2.0 '')	T.I. (0.75 cc)	71	61	39	25	18	11	9	6
5.	III-3 (2.0 '')	S.B.I. (0.75 cc)	75	92	100	108	194	220	150	45
6.	PRO + buffer (No Ca or III-3)		800	610	555	360	210	125	58	12
7.	III-3 + Ca (No PRO.)		78	—	—	—	—	—	87	—

unit TRYP.), as in mixture B. The lysates from these mixtures, as used in the subsequent prothrombin activation studies, obviously represent very small amounts of proteolytic enzyme.

Tryptic activation of prothrombin (Table I, B). Thrombic mixtures (T) consisting of 2 cc PRO.-A, 0.25 cc Ca, and cited activators (to 5 cc vol.) are sampled after successive incubation periods (i.t.), the 0.25 cc samples being added to 0.5 cc fibrinogen (B.F.) and the clotting-times (c.t.) noted. The shortening c.t. denotes increasing amounts of thrombin formation. This method has long been used in our laboratories for quantitative and qualitative study of the first phase of blood-clotting.[8]

The ¼ min. test, in all series, shows the trace of thrombin originally present in the particular prothrombin solution used. It is of very little significance even compared to the very slow and incomplete activation by Ca-salt alone, as shown in T_1. Series T_3 and T_4, on the other hand, show a definite "thromboplastic" acceleration of thrombin formation by the lysates containing tryptase (A) and trypsin (B), respectively. To rule out the possibility that H.F. might contain some thromboplastic factor other than tryptase, mixture C was prepared by simple heat (56°C) defibrination. Series T_2 corresponds closely to the T_1 control.

Other data. STREP. *(streptokinase)*, itself, in repeated tests, is found to be devoid of proteolytic, thromboplastic, or thrombic effects. Several other protease-containing plasma products, some of dog and bovine, as well as human, origin, also show the "thromboplastic" action noted in Table I, B. One Harvard human plasma fraction (III-3) is a potent protease but also possesses thrombic activity. This complicates the experimental analysis but, with appropriate controls, serves to show essentially the same phenomena, as illustrated in Table II. Included in these data are some typical results of the addition of known *trypsin-inhibitors* (T.I. and S.B.I.)

Inhibition of "thromboplastic" action of plasma protease III-3 (Table II). PRO.-B, used in these tests, forms thrombin "spontaneously" (T_6) at a somewhat greater rate than the purer product (PRO.-A), and Ca (T_1) gives maximal activation in 7 days, the 4-sec. clotting-time ultimately reached being identical with the stable value attained in 10-15 min. in the additional presence of brain thromboplastin (T_2). The protease III-3 is markedly "thromboplastic" (T_3), as shown by comparison with controls T_1 and T_7. The last shows only a weak initial thrombic action of III-3 alone which, if anything, is lessened after 24 hr. Pancreatic trypsin-inhibitor (T.I.) is clearly (T_4) able to inhibit a considerable amount of the thromboplastic effect of the protease. Soybean trypsin-inhibitor (S.B.I.) is even more effective (T_5), but the later tests suggest that part of this inhibition may be directed not only against the protease (III-3) but also against the natural thromboplastic factors undoubtedly operating in T_1 and T_6.

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[8] Ferguson, J. H., *Ann. N. Y. Acad. Sci.*, 1947, in press.

Fibrinolysis, etc. Lysis of the fibrin clots in A and B (Table I, A) occurred overnight, as did T_2 (Table I, B) and T_3 (Table II). This confirms the fibrinolytic actions of natural plasma protease. Fibrinolysis was absent, even after 7 days at 37°C, in the enzyme-free controls and in the presence of S.B.I., while it was delayed for 3-4 days by the cited amounts of T.I. Thrombinolysis requires further study, but did not occur with the proteases used in the present study, except trypsin (Table I, T_5). This could be a matter of enzyme strength, however.*

The absence of significant prothrombinolysis (*cf.*[9]), due to choice of very weak enzyme preparations, is apparently the key to the successful demonstration of these important "thromboplastic" effects.

Summary. The natural protease *(tryptase)* in several plasma protein fractions resembles a weak pancreatic trypsin in its fibrinogenolytic, fibrinolytic, and "thromboplastic" effects. These actions are inhibited by crystalline trypsin-inhibitors from pancreas and soybean. The clear demonstration of thromboplastic action in these quantitatively-controlled tests is direct proof of participation of plasma tryptase in the blood-clotting system.

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* More recent tests show that a sufficiently potent tryptase is thrombinolytic.

[9] Seegers, W. H., and Loomis, E. C., *Science*, 1946, **104**, 461.

15776

Chronic Oral Toxicity of Alpha-Naphthyl Thiourea.*

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Richter[1] proposed the use of alpha-naphthyl thiourea (usually abbreviated ANTU) as a specific poison for the control of Norway rats because of its high toxicity to rats and its relatively low toxicity to all other species tested. Its emetic property protected dogs in most cases. Although Richter showed that animals became tolerant to acute doses of ANTU, the experiments of McClosky and Smith[2] indicated a cumulative action with successive doses to rabbits and cats. In experiments conducted for short periods of time the chronic effects of ANTU paralleled closely those obtained with thiourea and 2-thiouracil.[1] Because of Richter's report of tolerance to ANTU the present study was undertaken to determine the effects of small amounts of ANTU ingested during the lifetime of the rat.

Experimental. Groups of 18 male weanling rats (21 days) from our colony of Osborne-Mendel strain were started on each of 7 diets containing respectively 0, 50, 100, 200, 400, 600 and 800 ppm ANTU. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ANTU was mixed with the basic diet by means of a rotary batch mixer. Litter mates were selected and assigned to the various groups according to a randomized design of experiment (balanced incomplete blocks).[3] All

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* A portion of the funds used in this investigation was supplied by a transfer between the Committee on Medical Research of the Office of Scientific Research and Development and the Division of Pharmacology, Food and Drug Administration, and also between the Office of the Surgeon General, War Department, and the Division of Pharmacology.

[1] Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

[2] McClosky, W. T., and Smith, M. I., *Pub. Health Rep.*, 1945, **60**, 1101.

[3] Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural, and Medical Research*, Oliver and Boyd, Edinburgh, 1938.

TABLE I.
Mean Gain in Weight of Male Rats Fed Diets Containing ANTU.

Time in mo.	Dosage of ANTU, ppm	No. of animals	Mean gain in wt, g	Standard error of mean, g
3	0	18	324.5	± 8.3
	50	17	315.1	± 7.3
	100	18	262.0	± 9.0*
	200	18	202.9	± 7.7*
	400	17	123.2	± 8.2*
	600	18	105.1	± 7.9*
	800	16	101.8	± 8.1*
12	0	12	498.1	±16.0
	50	16	505.8	± 7.2
	100	15	457.7	±22.9
	200	15	416.3	±15.2†
	400	16	260.6	±28.8*
	600	15	248.5	±11.4*
	800	12	240.3	±14.3*

* $p < .001$
† $p < .01$

animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals. Surviving animals were sacrificed at the end of the 2-year period.

Results. Effects on Growth and Mortality. Growth curves of the experimental groups, with the exception of that for the group on 50 ppm ANTU, deviated markedly from that of the controls. The data on the gains in weight during the fast-growing period of the first 12 weeks (Table I) show that the animals on concentrations of 100 ppm, or more, grew significantly ($p = < .001$) less than those on the control diet. When the gains in weight of the experimental and control animals during the first year were analyzed (Table I), the difference between the animals on 100 ppm ANTU and the controls was not significant ($p = .17$). This fact led to a further analysis of the growth data for the last 26 weeks of the first year. During this period all groups of rats on 100 ppm, or more, ANTU grew slower than the control group; however, the differences in growth between the groups on 100, 200 and 400 ppm ANTU and the controls were not significant. The growth rate of the rats on these concentrations of ANTU had increased so that the value for the retardation of growth had changed from highly significant at 3 months to nonsignificant during the plateau period. This apparent decrease in toxicity undoubtedly was due in part to the development of a tolerance;[1] however, as we have shown in a similar experiment with 2,2-bis (*p*-chlorophenyl)-1,1,1-trichloroethane (DDT),[4] it was due in part to the lesser daily intake of ANTU per kg of body weight as the animals increased in size. The reduction in dosage per kg of body weight was produced by the rapid increase in weight of the rats during the fast-growing period without a corresponding increase in food intake. A dislike for the food containing ANTU was evident in the rats on dosages from 400 to 800 ppm. For the first 3 or 4 weeks on the experimental diet these rats ate less per kg of body weight than control rats; later their food intake gradually became normal. After the first 3 months rats on dosage levels of 600 and 800 ppm were consuming daily more ANTU than the acute lethal dose of nontolerant rats of the same age.

All the deaths, except 4 in the group of rats on 800 ppm ANTU, which occurred during the first year of the experiment were caused either by respiratory infections or by middle ear disease. The 4 deaths in the group on 800 ppm appeared to be a direct effect of the ANTU; the animals were small and showed other signs of ANTU poisoning. At 18 months, although fewer animals were living in each group on 200 ppm, or more, ANTU than in the control group, no group had a death rate significantly different from that of the control. At the termination of the experiment the differences appeared to have a definite break at 200 ppm; however, because of the small number of animals the differences at 200 and 800 ppm ANTU were not significant. When the 4 groups from 200 to 800 ppm ANTU were compared jointly with the 3 groups which had an apparent normal death rate, namely, the groups on 50 and 100 ppm ANTU and the control, the difference in mortality rate was found to be highly significant.

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[4] Fitzhugh, O. G., and Nelson, A. A., *J. Pharm. Exp. Therap.*, 1947, **89**, 18.

Pathology. Autopsy was done on nearly all the 126 rats, and from 83 of them hematoxylin-eosin stained paraffin sections of various tissues were made for microscopic study. Lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal, testis and thyroid were sectioned routinely. In addition, sections of the following structures were obtained from the number of animals given: Parathyroid, 39; bones and muscles of knee joint region, 31; skin, 11; lymph nodes, 10; deformed leg and/or foot, 6; eyelids, 6; urinary bladder, 3; brain, 2. Perls' reaction for ferric iron was used on a number of sections to check the nature of certain pigments.

Externally, the rats were stunted and showed spectacle eyes, thinning and coarsening of the hair with increased prominence of the guard hairs, and deformities of the legs and feet (Table II). Internally, there were no characteristic changes, but some nonspecific ones were seen, in addition to those resulting from inanition. Slight or moderate relative or absolute enlargement of the spleen was noted 14 times, most frequently (6 instances) at 200 ppm. Dark semifluid material in the stomach or dark spots on the glandular stomach mucosa were noted 28 times, which together with 4 additional instances of focal hemorrhagic necrosis discovered microscopically make up the 32 listed

TABLE II.
Incidence of the More Frequent Pathological Findings.

Dosage ANTU ppm	No. of animals	Gross findings					No. examined microscopically	Microscopic findings			
		Emaciation	Spectacle eye	Hair loss	Leg deformity	Focal stomach hemorrhages		Hyaline liver	Calcified casts in kidney	Thyroid hyperplasia	Splenic hyperplasia
800	18	13	14	9	2	9	15	5	10	9	7
600	18	8	13	8	3	8	13	5	7	7	8
400	18	7	15	13	3	9	12	2	2	4	8
200	18	5	12	2	0	3	12	3	1	2	7
100	18	1	2	0	0	1	10	0	0	1	2
50	18	1	0	0	0	1	15	1	1	1	5
0	18	1	0	0	0	1	6	0	0	0	0

in Table II. In 7 animals, 5 of which were in the 800 and 600 ppm groups, the thyroid showed questionable or slight generalized enlargement.

The hair changes were most noticeable on the nose and on the rear half of the body and were related in degree to the dosage of ANTU. The cessation of hair growth was present in all rats surviving for the first year on 400 to 800 ppm ANTU and in 2 rats on 200 ppm. Spectacle eyes occurred in all rats on dosages from 200 to 800 ppm ANTU. Changes around the eyes were noticed first in animals on 800 ppm ANTU after about 6 months on the experimental diet, and later they occurred in the animals on lower dosage levels. The 3 instances of spectacle eyes in rats on 100 ppm ANTU appeared after 80 weeks. Sections of skin and eyelids showed mainly atrophy of the various structures. The epithelium was reduced in thickness. Hairs and sebaceous glands were reduced both in number and size. Chronic inflammatory cellular exudate was present in some of the eyelid sections, but not in the skin sections.

Hyperplasia of the thyroid was present microscopically (as judged from increased height of the epithelium and decreased amount of colloid) in about half the animals on 800 and 600 ppm ANTU, and in decreasing frequency at lower dosage levels, as shown in Table II. All the 24 instances of thyroid hyperplasia were of slight degree, except for 3 of slight-moderate and 1 of moderate degree. In a few instances there were papillary ingrowths and heapings of the follicular epithelium which might indicate that the present slight degree of hyperplasia had been somewhat greater at some time in the past.

Microscopic hyperplasia of the splenic pulp (Table II) was of moderate degree in 13 instances and slight in the remaining 24, with the moderate degree more frequent at the higher than at the lower dosage levels. It is probable that parallelism of splenic hyperplasia with dosage of ANTU is not more marked because of counteraction of hyperplasia at the higher dosage levels by inanition, which tends to cause hypoplasia (atrophy) of the spleen. In 24 instances, all at 200 ppm or above, a relative increase in the percentage of myeloid cells in the splenic pulp was noted; these spleens included atrophic as well as hyperplastic ones.

Hyaline change in the cytoplasm of the centrolobular hepatic cells (Table II) was noted in 16 instances, all graded as slight or minimal in degree except for 2 of moderate and 1 of slight-moderate degree. The lesion consisted of increased oxyphilia and slight to moderate hyalinization of the cytoplasm of the centrolobular hepatic cells, with a minimal increase in cell size. It resembled the lesser degrees of the typical lesion in the rat liver caused by the feeding of DDT,[5] but did not show the peripheral segregation of large basophilic cytoplasmic granules and the distinct increase in cell size present in many of the DDT rats. Otherwise, we have not previously noted this lesion. Two rats showed focal necrosis of the liver, an incidence to be expected even in untreated animals. There was no vacuolation of the hepatic cells to indicate hydropic or fatty degeneration.

A slight or even moderate decrease in the thickness of the adrenal cortex was seen in most animals at the higher dosage levels, and less frequently with decreasing dosage level through the 200 ppm group; it was not distinct below that level. There did not appear to be a decrease in the number of cortical cells; the cells were simply smaller than usual, having lost most of the foaminess of the cytoplasm normally present in greater or lesser degree. Fat stains were not done. This change has not been noted previously in simple chronic inanition. The adrenal medulla was not altered.

Calcified tubular casts were found in the renal medulla in very small to moderate numbers (numerous in 2 instances at 800 ppm) and their incidence was proportional to dosage, as shown in the table. They first appeared at 35 weeks. They caused little or no renal tubular destruction. The kidneys otherwise showed nothing of note.

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[5] Nelson, A. A., Draize, J. H., Woodard, G., Fitzhugh, O. G., Smith, R. B., Jr., and Calvery, H. O., *Pub. Health Rep.*, **1944**, **59**, 1009.

Foci of superficial terminal hemorrhagic necrosis of the glandular mucosa of the stomach are rather frequently seen in rats found dead, especially after a short period of treatment (*i.e.*, with a highly toxic agent). However, the incidence (Table II) in ANTU rats at the higher dosage levels was greater than usual, and the lesions were found in sacrificed animals as well as in those found dead. A sharp drop in incidence below 400 ppm will be noted. The lesion was generally not massive.

Bone changes (upper half of tibia and lower half of femur), apart from the peculiar deformities noted below, consisted in an increase in the number of trabeculae of spongy bone, an increase to a lesser extent in their thickness, and irregularity and slight thinning of the cortical bone. Sometimes a small portion of the spongy bone had the appearance (increased oxyphilia and decreased density) of being newly formed. In some animals small to moderate amounts of fibrous tissue surrounded some of the spongy bone and encroached on the marrow. Few osteoblasts or osteoclasts were seen, suggesting a slow tempo or chronic nature of these changes.

The changes in the 31 bone marrows sectioned were complicated by the associated ones in the bone, but generally speaking slight myeloid hyperplasia was frequent at the higher dosage levels, less frequent at 200 ppm, and absent below that level. On the whole the marrow in the epiphyses was not as hyperplastic as that in the shafts. The remarks about inanition in connection with the spleen also apply here. The ultimate cause of the hyperplasias in the spleen and bone marrow is uncertain.

Deformities of the legs and feet were noted after the 40th week in 8 animals receiving 400 or more ppm ANTU. There were contractures of the extremities, so that the affected animals had the appearance of walking on their heels and elbows, combined with twisting of the feet or legs; sometimes the paws would face backwards. Chronic granulomas of the feet were more severe and the incidence was distinctly higher in treated animals than usually have occurred in similar experiments with other substances.

Structures unchanged from their appearance in the controls by the feeding of ANTU were lung, heart, lymph nodes, proventriculus, small intestine, colon, and parathyroid. Pancreas and testis differed only in showing nonspecific changes from inanition. Pigmentation in spleen and adrenal was as generally seen in adult rats. The incidence of middle ear infection, chronic pneumonia, and various spontaneous tumors was not increased.

Discussion. Both the hair changes and the spectacle eyes generally are considered to be caused by nutritional deficiencies. Richter[1] has reported that feeding cystine prevents the cessation of hair growth. Circumocular alopecia, or the "spectacle eye condition," has been reported to occur in biotin deficiency[6] and in inositol deficiency;[7] however, this condition often is associated with dermatitis elsewhere and is not necessarily a sign of a single deficiency of a member of the vitamin B complex.[8] Many of the rats on the higher dosages of ANTU showed inanition to a marked degree, and therefore complex nutritional deficiencies were probably present.

Destruction of bone and joint tissues in the feet and lower legs by suppurative inflammation and the reactive processes consequent thereto appeared to be the underlying cause of the deformities of the legs and feet. The subsequent contractures and twisting are accounted for in the same way that they occur in human cases unless they are prevented. The chronic granulomas of the feet, which are occasionally seen in our rats, presumably resulted from the trauma of the cage wires. It seems reasonable to assume that the various nutritional, endocrine and osseous upsets produced by feeding ANTU caused the tissues of the feet to be a more favorable location than they are usually for bacterial growth. Except where involved by adjacent inflammatory processes, the muscles showed surprisingly little change. The lack of muscle changes rules out to some extent a vitamin E deficiency.

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[6] Nielsen, E., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 349.

[7] Pavcek, P. L., and Baum, H. M., *Science*, 1940, **92**, 502.

[8] Sullivan, M., and Nicholls, J., *Arch. Der. and Syph.*, 1942, **45**, 917.

Summary. When ANTU was fed chronically to rats for 2 years in concentrations of 50 to 800 ppm in the diet, the concentrations from 100 to 800 ppm were toxic. Externally, the rats were stunted and showed spectacle eyes, thinning and coarsening of the hair and deformities of the legs and feet. Histopathological changes consisted of hyperplasia of the thyroid, hyperplasia of the splenic pulp, hyaline changes in the cytoplasm of the centrolobular hepatic cells, a slight to moderate decrease in the thickness of the adrenal cortex, calcified tubular casts in the renal medulla, slight myeloid hyperplasia of the bone marrow, and slight modifications in bone structure. The degree of injury in each case was related to the concentration of the ANTU. The concentrations of 600 and 800 ppm ANTU produced a marked tolerance.

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Separation of Two Mutually Antagonistic Chromatophorotropins from the Tritocerebral Commissure of *Crago*.

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From the time of Koller's original contention that he had discovered in the rostral region of the thorax of the shrimp, *Crago,* a new endocrine source, producing a principle which blackened white-adapted specimens,[1] repeated attempts to confirm this conclusion have been generally unsuccessful at the hands of other investigators. More recently it has been shown that the circumoesophageal connective region,[2] or more specifically the tritocerebral commissure, of *Crago*[3] possesses very potent chromatophorotropic activity. Extracts of the commissures in sea-water, injected into eyestalkless specimens produce within 5 minutes striking lightening of the body and simultaneously blackening of the tail-fin (telson and uropods). It has been postulated upon the basis of such observations as the following, that the tritocerebral commissure of *Crago* possesses 2 mutually antagonistic principles for general body-coloration: (1) extracts of commissures from different individuals show variation in their relative influences in body-lightening and tail-fin-darkening, at times even darkening the body instead of lightening it,[3] (2) stimulation of the stubs of the eyestalks in eyestalkless animals results in body-lightening and tail-fin-darkening when the stimulus is strong, and, overall blackening if the stimulus is weak,[4] (3) comparative studies of crustacean nervous systems show some to possess only body-lightening activity (*e.g.* the crab, *Uca*), others predominantly body- and tail-fin-darkening activity (*e.g.* the lobster, *Homarus*).[5]

A clear proof, however, that the observed results of the action of extracts of *Crago* tritocerebral commissures are due to 2 hormones (1) a body-blackening hormone, and (2) an antagonistic body-lightening one obviously requires the chemical fractionation of the *Crago* commissure into 2 portions, each possessing only one of these 2 actions.

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[1] Koller, G., *Z. vergl. Physiol.*, 1928, **8**, 601.

[2] Brown, F. A., Jr., and Ederstrom, H. E., *J. Exp. Zool.*, 1940, **85**, 53.

[3] Brown, F. A., Jr., *Physiol. Zool.*, 1946, **19**, 215.

[4] Brown, F. A., Jr., and Wulff, V. J., *J. Cell. Comp. Physiol.*, 1941, **18**, 339.

[5] Brown, F. A., Jr., and Saigh, L. M., *Biol. Bull.*, 1946, **91**, 170.

TABLE I.
Fractionation of Principles of *Crago* Commissure by Drop Method.
Absolute Ethyl Alcohol as Extracting Solvent.

Extraction No.	No. of drops of solvent	Quantity of sea water to take up residue cc	Size of injection, cc	Assay	
				Body	Tail
1	5	.06	.03	—4	0
2	5	.06	.03	—4	0
3	5	.06	.03	—3	0
4	5	.06	.03	—1	0
5	5	.06	.03	—1	0
6	—	.06*	.03	—2 i† +4 f	+4

* Water was added directly to the slide and the smeared tissue was scraped off and thoroughly mixed with the liquid.
† i means initial; f means final.

TABLE II.
Fractionation of Principles of *Crago* Commissure by Equilibrium Method.
Absolute Methyl Alcohol as Extracting Solvent.

Extraction No.	Quantity of solvent in test tube, cc	Quantity of sea water to take up residue, cc	Size of injection, cc	Assay	
				Body	Tail
1	.50	.06	.03	—3	0
2	.50	.06	.03	0	0
3	—	.06	.03	—1 i†	+4
1	.50	.06	.03	—3	0
2	.50	.06	.03	—1	0
3	.50	.06	.03	0	0
4	.50	.06	.03	0	0
5	—	.06*	.03	0 i† +1 f	+3

* Water was added directly to the slide and the smeared tissue was scraped off thoroughly and mixed with the liquid.
† i stands for initial; f stands for final.

Investigations of the solubility of the active commissural material in organic solvents[3] indicated that one of the factors is preferentially soluble in polar organic substances, such as ethyl alcohol. A fractionation method based on an extraction procedure was thus suggested. Craig has shown[6] that the method of successive extractions can be used very successfully in fractionating substances of biological interest. Unfortunately his procedure deals with quantities of the order of 20 μg or more. A rough measurement of the size of the glandular tissue of the commissure as determined from histological serial sections indicated that the quantity of active principle available must be much smaller than even one microgram. Considering the region of the commissure possessing the gland as approximately a cylinder, one finds for an average specimen a diameter of about 0.005 cm, a length of about .05 cm, and hence a volume of about 1×10^{-6} cc. Assuming a density of about 1, one obtains 1×10^{-6} g for the mass of this structure. A very generous estimate of the actual glandular tissue in the commissure would be about 25%. The concentration of active substance within the glandular tissue could hardly exceed 33% so that a mass of 0.1 μg is probably an upper limit to the total quantity of active material present. It is this 0.1 μg which must be fractionated into 2 components.

Experimental. A commissure was carefully excised from a medium sized (1 g) specimen of the shrimp, *Crago,* as previously described[3]

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[6] Craig, L. C., *J. Biol. Chem.*, 1944, **155**, 519.

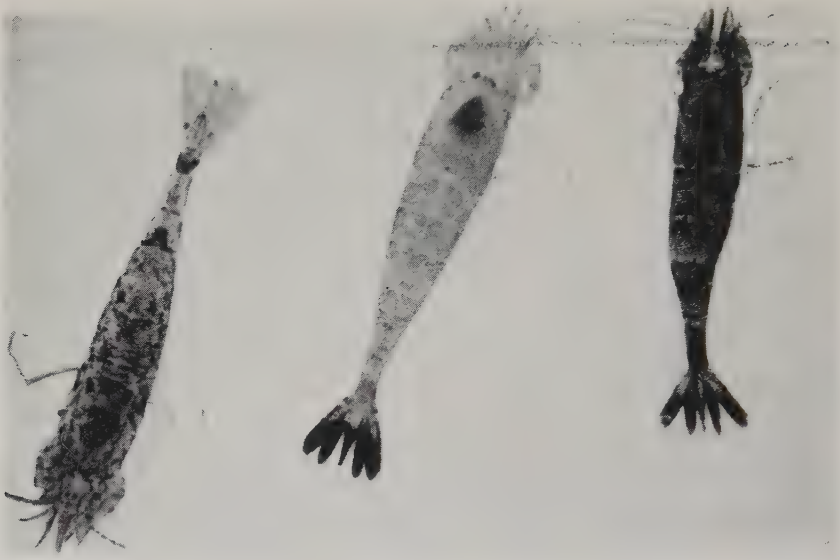

FIG. 1.

From left to right: 1. A sea-water-injected eyestalkless *Crago* (control); 2. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the sea-water-soluble contents of one tritocerebral commissure; 3. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the alcohol-insoluble contents of a tritocerebral commissure. The injections were made about nine minutes before the photograph was taken. All 3 animls were matched and resembled specimen one at the beginning of the experiment.

and deposited on a microscope slide or cover-glass. The surrounding water was allowed to dry for about 2 minutes. The specimen was then smeared over an area of about 0.5 cm^2 with the aid of a blunt glass rod or cover-glass. Examination under the microscope indicated that the maximum thickness of the smeared specimen was about 5 μ and that numerous valleys and ridges were present to facilitate penetration by the solvent.

Two alternative procedures were used to carry out the extraction. One consisted of dropping the organic solvent onto the tilted slide bearing the smeared specimen and collecting the liquid on a depression slide. The liquid was collected in groups of 5 or 10 drops. An interval of a few minutes was allowed between each group so that the specimen slide could dry.

This procedure was found to have the objectionable feature, particularly with very volatile solvents, that rapid evaporation of the solvent cooled the slide appreciably and led to the condensation of water vapor on the specimen. The water could dissolve both active principles and hence nullify the effort at fractionation. This difficulty was overcome at least partially by drying between groups of drops, but some doubt always remained.

An additional objection to this drop procedure lies in the difficulty of carrying out a physico-chemical analysis of the results since the establishment of equilibrium between smear and solvent can be seriously questioned.

To overcome the objections mentioned a modified approach was also used. The cover-glass containing the smear was cut down to approximately the area occupied by the tissue and then inserted into a 3 cc test tube. 0.50 cc of solvent was added to the tube, which was then stoppered and shaken carefully for about 10 minutes. The closed tube minimized evaporation and the long time of contact between tissue and solvent insured the attainment of equilibrium.

With either procedure the solvent was evaporated in the open air and the residue was taken up in sea water. The sea-water solution was used in all injections.

The method of assay has been described previously. A scale from 0 to 4 has been employed to estimate the intensity of a given effect. A negative sign indicates a lightening with respect to an appropriate control, a positive sign indicates a darkening.

Results. A typical experiment using the drop technic is summarized in Table I and an example of the test-tube method is illustrated in Table II. In both cases it is quite clear that the body-lightening factor (CBLH) can be separated without any significant amount of the tail-darkening principle (CDH). The solvent used in the experiment of Table I, however, is not exceedingly volatile. Using isopropyl alcohol as the extracting solvent, a cleaner separation was effected. With methyl alcohol, which vaporized very rapidly, assays usually show a slight amount of tail-darkening in the collected drops of solvent. It was quite obvious that water was condensing on the slide during the dropping of the methyl alcohol and the water must have carried along a little of the tail-darkening factor, for when the closed-tube technic was adopted (Table II) none of the darkening factor appeared in the alcohol extracts. Apparently the darkening factor has no significant solubility in the organic solvents used.

Fig. 1 illustrates the action of a sea-water extract of a tritocerebral commissure which contains both CBLH and CDH, and the action of only the alcohol-insoluble fraction of the commissure which contains CDH alone.

Summary. The tritocerebral commissure of the shrimp, Crago, has been chemically fractionated to separate 2 mutually antagonistic chromatophorotropins influencing body-coloration, a general darkening one (CDH) and a body-lightening one (CBLH).

15778

Radioautographs in Which the Tissue is Mounted Directly on the Photographic Plate.

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In early methods of determining the location of radioactive elements in tissue, the microscope slide bearing the tissue section was placed face down on a photographic plate.[1-4] After a suitable exposure to the radiation the plate was removed and developed. The resulting image, or autograph, was then compared with the tissue section (after staining etc.) to locate the radioactive material. The efficacy of this method is limited by (1) lack of sharpness and (2) difficulty in matching the autograph with the corresponding histologic detail in the tissue.

Recently, Bélanger and Leblond[5] have described a method which reduces these objections. The photographic emulsion from a lantern slide is removed and spread over the tissue section. After the radiation exposure the autograph is developed, fixed and washed. The tissue sections are then stained, cleared and the entire preparation mounted in balsam.

For some time the writer had been considering the feasibility of mounting tissue sections directly on the photographic emul-

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[*] It is a pleasure to acknowledge the assistance and encouragement given to the writer by his associates in the laboratory.

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[2] Hamilton, J. G., Soley, M. H., and Eichorn, K. B., *Univ. California Publ., Pharmacol.*, 1940, **1**, 339.

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[4] Hamilton, J. G., *Radiology*, 1942, **39**, 541.

[5] Bélanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, **39**, 8.

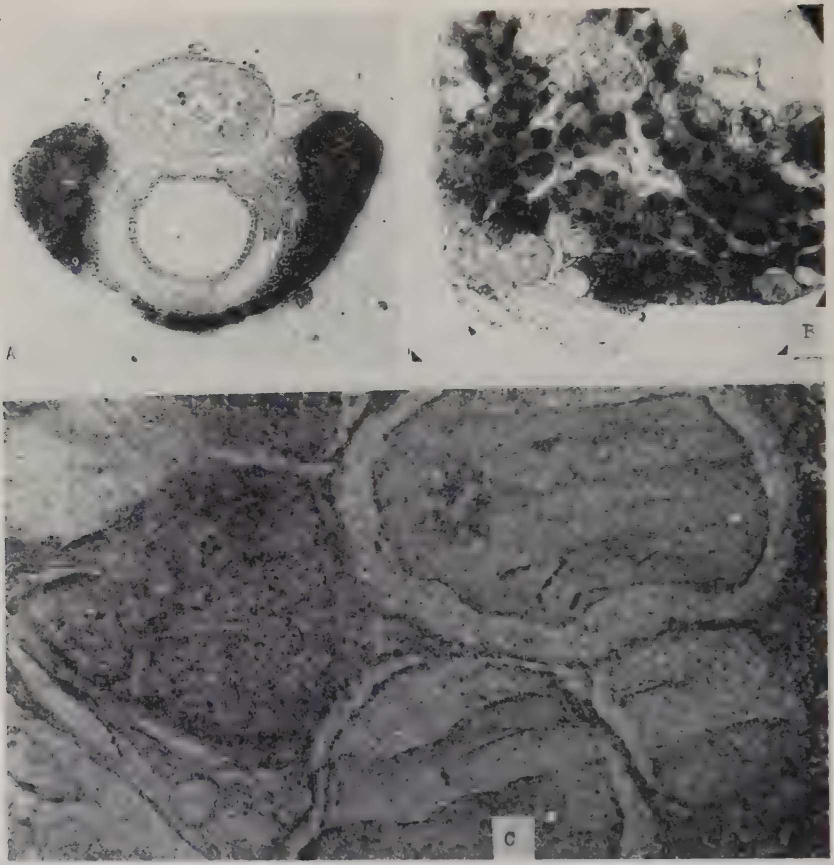

PLATE 1.

Photomicrographs of rat thyroid tissue (hematoxylin and eosin) mounted on the photographic emulsion in which the autograph was later produced directly under the regions containing radioiodine.

A. Thyroid region of newborn rat. Autograph and tissue on commercial film. Magnification ×18. Autograph developed 4 days after tissue containing the radioiodine had been attached. Three days before this babe was born, the mother had been injected with 30 μc of I^{131}. The foetus was able to concentrate the iodine in its thyroid, in competition with the mother, as can be seen by the heavy blackening which is limited to the thyroid regions.

B. Portion of thyroid of young adult rat (wt. 171 g) which had been sacrificed 24 hours after an injection of 25 μc of I^{131}. Magnification ×25. Medium contrast lantern slide, 6-day exposure. The indication of presence of radioiodine is most pronounced in the smaller follicles.

C. Small region of same thyroid shown in B, ×188. Deposition of the reduced silver is heavy underneath the one triangular follicle, whereas that under the others is only slightly greater than background (*i.e.*, lower left corner).

sion. When the paper of Bélanger and Leblond appeared, their method was tried. The preparations, although better than those obtained with the older methods, were not completely satisfactory. The emulsion was uneven and contained bubbles, the autograph was not sharp enough for high power study, the "fog" was heavy, and staining was complicated by the gelatin over the tissue, which became colored and lifted away from the slide. No doubt, some of the difficulties were due to inexperience and would be eliminated in time. It seemed advisable, however, to try our first plan, *i.e.* to mount the tissue directly on the plate. This method has been satisfactory and gives much better resolution than was possible formerly.

Fixation, dehydration, embedding and sectioning are done according to the usual histologic technics. The paraffin ribbons are then cut into strips of 2-4 sections and allowed to expand on the surface of water at 42°C in a small petri dish. This is dropped into a larger bowl which allows the sections to float out into cold water. The photographic plate (lantern slide, or film not sensitive to red light) is then slipped under the tissue sections in dark room, in front of red safelight, one corner of the section is held against the emulsion of the plate and the plate plus the tissue removed from the water. The preparation is then dried in front of a fan and placed in a light-tight box for exposure to the radiation. Before developing the photographic plate the paraffin is removed with xylol and when this has evaporated the plate is developed in Eastman D-11 or D-72 solution. After fixation in acid hypo, the preparation is washed and allowed to dry. If it is desired that the background (and also the image) be reduced, this can be done by dipping the preparation, before the tissue is stained into a 0.2% permanganate solution.[6]

The tissue is stained with Harris' hematoxylin (over stain, acid water, alkaline water) and aqueous eosin, then dehydrated, cleared and mounted in permount or balsam. The plate should be dried as often as possible. Swelling and reticulation of the emulsion must be avoided.

Several methods of avoiding heavy staining of the photographic film, which is more of a problem than when plates are used, have been employed successfully. One method is staining *in toto* (acetocarmine, Harris' hematoxylin, or eosin when absolute alcohol is the fixative). Another, is to place ferric alum in with the fixative[7] in order that the tissue may be selectively stained when the preparation is immersed in iron hematoxylin. Care must be exercised in the second use of ferric alum since it will reduce the autograph as well as the nuclear stain.

Tissue prepared in the usual way may be used to check detail and staining properties. Some of the tissue should also be used for autographs using the older method, to insure that the general distribution of the radioactive material appears to be the same by both methods.

Tissue-autograph preparations offer many advantages over the older method of preparing the two separately. The entire section may be studied simultaneously for histologic detail and for localization of the radioactive element. The resolution is better and studies can be made under either the low or high power of the compound microscope (Plate 1). These findings have aided materially the study of experimental animals, of thyroid adenomas, and of thyroid carcinoma metastases.

Summary. The tissue section containing radioactive material is mounted directly on the photographic emulsion in the dark room. After suitable exposure, the plate is developed, and the tissue stained etc. The preparation may be studied microscopically as the autographic image is in place just below the tissue.

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15779

Storage, Retention and Distribution of Folic Acid in the Chick.*

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The water-soluble vitamins are generally stored and retained by animal tissues to a much smaller extent than the fat-soluble vitamins. High oral intakes of vitamin A are known to produce high concentrations of this vitamin in the livers of rats and once established these stores are available to the animal for long periods of time following the exclusion of vitamin A from the diet.[1] Data on the storage and retention of the B vitamins are limited. Schultz *et al.*[2] were unable to find a further increase in the body concentration of thiamine after a dietary level of 65 γ of thiamine per rat per day was reached. Pearson *et al.*[3] found that an increased dietary level of pantothenic acid had no significant effect on the level of this vitamin in the livers of chicks but did produce increases in the leg and breast muscle.

In the course of our studies on folic acid in chick nutrition we were interested in the storage, retention, and distribution of this vitamin in the chick. Results of these investigations are reported here.

Experimental. Day-old White Leghorn cockerels obtained from a commercial hatchery were maintained in electrically-heated cages with raised screen bottoms. All chicks were fed our folic acid-deficient basal ration 494-K which consists of dextrin 61 g, alcohol-extracted casein 18 g, gelatin 10 g, soybean oil 5 g, salts V 6 g, cystine 0.3 g, thiamine 0.3 mg, riboflavin 0.6 mg, nicotinic acid 5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, inositol 100 mg, choline chloride 150 mg, biotin 0.02 mg, 2-methyl-1,4-napthoquinone 0.05 mg, and α-tocopherol 0.3 mg. Each chick received in addition one drop per week of fortified haliver oil containing 120 A.O.A.C. units of vitamin D_3 and 1200 U.S.P. units of vitamin A per drop. Synthetic folic acid (pteroyl glutamic acid) was used in all experiments. Food and water were given *ad libitum.* The chicks were maintained on the above ration for 3 to 4 days, weighed, and chicks within a 10 g weight range were distributed uniformly into the experimental groups.

In the first series groups of 8 chicks each were placed on folic acid levels of 0, 25, 100, and 1000 γ per 100 g of ration. The number of chicks per group was reduced to 6 after the first week by discarding the heaviest and lightest animal from each group. The animals were sacrificed when 4 weeks old and representative samples of liver and muscle tissue were taken from 4 chicks in each group for folic acid analyses. Average weights of the animals at 4 weeks, number of survivors, and folic acid values for the liver and muscle tissues are summarized in Table I.

In a second series, 4 groups of 16 chicks each were placed on the following diets: unsupplemented basal, basal plus 200 γ of folic acid per 100 g of ration, basal plus 1000 γ of folic acid per chick per day for 5 days (total of 5 mg per chick) by intraperitoneal injection, and basal plus 1000 γ of folic acid per chick per day for 5 days orally. Four chicks from each group were taken for folic acid analysis of the liver and muscle tissues at the end of 1, 3, and 5 weeks from the time the supplements were started. In addition, folic acid analyses were made on representative tissues from 3 series of 4- to 5-week-old

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TABLE I.
Folic Acid Storage in Chick Liver and Muscle with Increasing Dietary Intakes of Folic Acid.

Group No.	Supplement added per 100 g ration 494-K	Avg wt at 4 wk, g	γ of folic acid per g of	
			Liver	Muscle
1	0	130 (6)*	1.07 (.79-1.52)†	.05 (.042-.069)
2	25 γ folic acid	175 (5)	2.35 (1.46-3.08)	.06 (.04-.07)
3	100 γ ” ”	190 (6)	3.52 (1.97-4.51)	.06 (.05-.08)
4	1000 γ ” ”	175 (6)	14.7 (11.9-16.4)	.04 (.03-.05)

* Numbers in parentheses refer to number of chicks surviving at 4 weeks.
† Numbers in parentheses give the range of values found.

chicks receiving 200 γ of folic acid per 100 g of our basal ration. The results of these series are summarized in Tables II and III.

Samples taken for analysis were minced in a Potter-Elvehjem homogenizer[4] or a Waring Blendor. Liver and muscle samples from chicks in series I and II were digested with taka-diastase in pH 4.5 acetate buffer for 24 hours at 37°C since this procedure has been found satisfactory for liberating bound folic acid in these tissues.[5,6] The other body tissues were likewise treated with taka-diastase and in addition duplicate series were treated in pH 4.5 acetate buffer with a kidney conjugase preparation prepared according to the procedure of Bird *et al.*[7] No significant differences in liberation of folic acid from the various tissues was noted with either procedure and the values given (Table III) represent an average of values from tissues receiving the 2 liberation procedures. Folic acid was determined with *Streptococcus faecalis* using the medium of Luckey *et al.*[8] modified by the addition of 1.0 cc of Speakman's[9] salts B per 100 cc of medium.

Results and Discussion. The results of the first series demonstrate that increasing the dietary level of folic acid has little effect on the content of this vitamin in chick muscle tissue since muscle folic acid values remained relatively constant regardless of dietary intake. The folic acid content of the liver, however, increased directly with increased dietary intakes of the vitamin. Liver values were 1.07, 2.35, 3.52 and 14.7 γ of folic acid per g of fresh liver for the 0, 25, 100 and 1000 γ dietary level groups respectively. The values presented here are not intended to represent absolute amounts of folic acid that will be found in chick liver and muscle tissue on similar folic acid intakes on different diets; but they merely represent the relative efficiency of storage of folic acid under the particular standardized conditions used in this series (Series I). The observed increase of folic acid in chick liver as compared to the constancy of chick muscle tissue follows the expected storage of a B vitamin and is in contrast to the increased storage of pantothenic acid in muscle tissue reported by Pearson *et al.*[3]

Results from the second series indicate that high initial dosages of the vitamin are stored inefficiently in the liver since only 3.6 γ of folic acid per g of liver were present in chicks from Groups 3 and 4 (Table II) 2 days after administration of the final mg of folic acid. Muscle folic acid values remained fairly constant in Groups 1, 2 and 4 but a temporary increase occurred in the injected group. The control group of chicks

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TABLE II.
Folic Acid Content of Liver and Muscle Tissue from Chicks Receiving Various Folic Acid Supplements.

Method and amount of supplementation	Avg weight and folic acid values in γ/g								
	1st week*			3rd week			5th week		
	Avg wt, g	Folic acid in Liver	Folic acid in Muscle	Avg wt, g	Folic acid in Liver	Folic acid in Muscle	Avg wt,‡ g	Folic acid in Liver	Folic acid in Muscle
0	85	.80 (0.4-1.2)†	.05 (.04-.06)	95	1.1 (0.7-1.1)	.04 (.04-.05)	85	1.0 (0.4-2.1)	.09 (.05-.13)
200 γ folic acid per 100 g basal diet	90	2.1 (1.2-2.7)	.05 (.04-.06)	215	1.6 (1.2-1.9)	.06 (.05-.08)	410	3.9 (3.6-4.3)	.07 (.07)
1 mg folic acid per day for 5 days by injection	90	3.6 (2.8-5.0)	.27 (.21-.34)	220	1.2 (1.2-1.7)	.09 (.07-.12)	395	0.6 (.45-.70)	.10 (.09-.16)
1 mg folic acid per day for 5 days orally	90	3.6 (1.4-5.0)	.08 (.06-.09)	210	0.7 (0.5-0.7)	.06 (.05-.07)	385	0.34 (.30-.40)	.12 (.09-.18)

* Weekly periods are given from the time supplementation was started.
† Numbers in parentheses refer to range of folic acid values.
‡ Number of deaths during the 5-week period was 1, 0, 0, and 0 for groups 1, 2, 3, and 4 respectively.

TABLE III.
Folic Acid Content of Tissues from 4-5-Week-Old Chicks.

Tissue	Folic acid in γ/g*
Liver	3.05 (1.8-4.3)†
Kidney	1.6 (1.0-2.2)
Pancreas	.95 (.4-1.5)
Testes	.6
Spleen	.28 (.19-.27)
Heart	.21 (.19-.23)
Brain	.19 (.17-.2)
Lung	.17 (.10-.24)
Muscle	.08 (.04-.12)
Skin	.05 (.05-.06)

* Each value represents the average of 6 samples, each of which consisted of the pooled tissue of 2 chicks (total of 12 chicks).
† Numbers in parentheses refer to range of folic acid values encountered.

which received the 200 γ level of folic acid had liver and muscle folic acid values in fair agreement with those obtained in the first series. The failure of the chicks on the 200 γ dietary level of folic acid (Table II) to show the expected increases in liver folic acid values over the groups receiving the 100 γ dietary level (Table I) suggests more efficient storage of folic acid at an early age in the slower growing animals of the first series. No significant decreases in liver folic acid occurred in the basal group and the 0.6 and .36 γ/g drop from the 3rd to the 5th week in Groups 3 and 4 respectively is small compared to the 2.4 and 2.9 γ/g drop that occurred from the 1st to the 3rd week. Further, the increased growth from the 3rd to the 5th week as compared to the 1st to the 3rd week would act to distribute the vitamin over a larger weight of tissue and the decreases in the total amount of liver folic acid would actually be smaller than is indicated by the values given. The folic acid in the basal ration (3 to 4 γ per 100 g) plus that produced from a limited amount of intestinal synthesis would aid slightly in maintaining liver stores. Nevertheless, the ability of the injected and orally administered groups to continue growing in spite of the fact that their liver and muscle tissues were as low in folic acid as the corresponding tissues of the basal group is interesting. Explanations for this discrepancy are lacking, but one possibility is that the initial high dosages of folic acid were used in the synthesis of a biologically active but microbiologically inactive compound. This view has been taken previously by Wright *et al.*[10] who observed a decrease of folic acid (as measured microbiologically) in incubated rat liver tissue. In any event, low tissue levels of folic acid are utilized efficiently on low folic acid diets. The possibility of storage in some other tissue of the animal seems improbable since analysis of the various body tissues of chicks receiving 200 γ of folic acid per 100 g of diet showed the liver to be the main site of storage.

Results from the second series also demonstrate that high initial doses of orally administered folic acid are used as efficiently as equivalent doses of injected folic acid. Group 4, which received the folic acid supplement by the oral route, gave a growth response at 3 weeks equivalent to that of the injected group (Group 3); and at 5 weeks the growth of the 2 groups plateaued at the same weight (difference of 10 g is not considered significant). These results do not substantiate the findings of Frost and Dann[11] who found differences in favor of the injected folic acid but a direct comparison of the data is impossible since different technics and animals were involved.

Liver, kidney, and pancreas were found to have the highest and skin and muscle the lowest content of folic acid; testes, spleen, heart, brain, and lung had intermediate values. The relative content of folic acid in chick liver, heart, and brain agree with the values found by Williams *et al.*[12]

Summary. High oral intakes of folic acid are stored inefficiently in chick livers and have little if any effect on the content of this vitamin in chick muscle tissue. Chicks given large oral doses of folic acid and then placed on a folic acid-deficient diet grew as well as chicks receiving an equivalent amount of folic acid by injection. The distribution of folic acid in some representative chick tissues has been studied.

We are indebted to Merck and Company, Rahway, N.J., for crystalline vitamins; to Dr. B. L. Hutchings of Lederle Laboratories, Inc., Pearl River, N.Y., for crystalline folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Company, Inc., Chicago, Ill., for gelatin; to Allied Mills, Inc., Peoria, Ill., for soybean oil; and to E. I. du Pont de Nemours and Company, Inc., New Brunswick, N.J., for crystalline vitamin D_3.

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15780

Intrasplenic Transplantation of Testes in Castrated Mice*

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Granulosa-cell tumors and luteomas have arisen in intrasplenic transplants of ovaries in castrated male and female mice.[1,2] The pathogenetic factors responsible for these ovarian tumors have been assumed to be (1) the capability of the liver to inactivate ovarian hormones, and (2) the increase in the amount of gonadotrophic hormones that

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* Aided by grants from the Anna Fuller Fund and the Jane Coffin Childs Memorial Fund for Medical Research.

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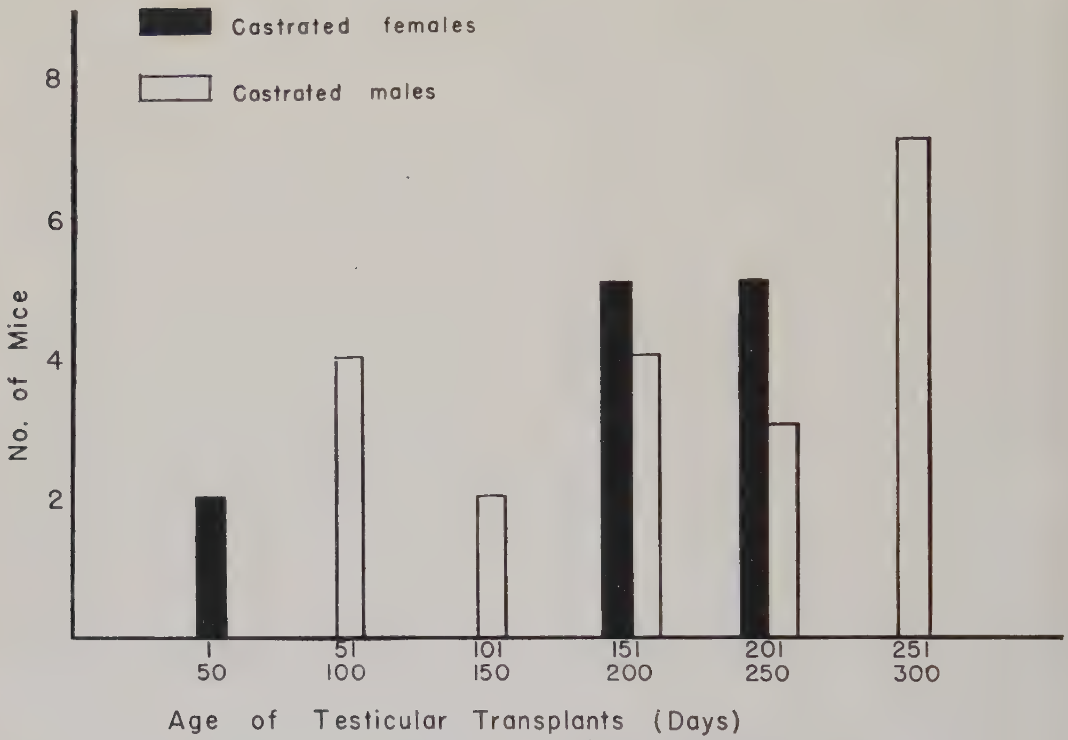

FIG. 1. Age of the intrasplenic testicular transplants in castrated male and female mice.

follows castration. A failure to obtain androgenic effects on the accessory genital organs of castrated male rats and rabbits occurred when androgens were administered so that they passed through the hepatic portal system before gaining access to the general circulation.[3-7] Biskind and Biskind[8] have reported that one granulosa cell-like tumor developed in an intrasplenic testicular graft in a castrated male rat. The fate of intrasplenic testicular transplants in castrated male and female mice and evidence for their endocrine effects are reported in this paper.

Materials and Methods. Inbred mice of the A strain were used.‡ Both male and female mice were castrated when they were 1 to 3 months of age, and a testis obtained from 2- to 7-day-old male mice of the same strain was transplanted into the spleen of each animal at the time of castration. The animals were fed on a commercial diet (Nurishmix), with occasional supplements of grain (a mixture of wheat, oats, sunflower seeds, and calf-meal pellets), and water. The age of the intrasplenic transplants of testes at the time of autopsy is given in Fig. 1.

Results. The spleen provided a good vascular supply for the testicular grafts; they increased in size shortly after the transplantation as observed at laparotomy. The transplants usually protruded from the surface of the spleen; the largest measured about 4 x 8 mm in diameter (Fig. 2).

Twenty testicular grafts were recovered from castrated male mice bearing intrasplenic transplants for a period ranging from 63 to

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‡ These mice were supplied by Doctors A. Gorbman, C. W. Hooker, and L. C. Strong.

275 days. Testicular grafts were not found, however, in 2 hosts sacrificed at 273 and 296 days after the operation. Microscopically, the testicular grafts were well incorporated with the splenic tissues and blood sinuses although major portions of the grafts were covered only by the splenic capsule. A few layers of proliferating spermatogonia and spermatocytes were observed in the seminiferous tubules of transplants recovered after 63 to 140 days. Sloughing of the spermatogenic cells into the lumina of the tubules, resulting in a disorganized appearance of the seminiferous epithelium, was frequently encountered. A slight increase in the number of interstitial cells of Leydig, as compared to a normal testis, was noted in 3 testicular grafts. Minute vacuoles were present in the cytoplasm of many of the interstitial cells. In testes grafted into the spleens for a period of over 160 days the seminiferous epithelium was generally thin, although the lumina of the seminiferous tubules remained large (Fig. 3). A few large primary spermatocytes containing 2 or more nuclei were noted in many of the tubules. The nuclei in these large primary spermatocytes were usually at the same stage of meiosis or were in a resting stage with a distinct nuclear membrane. Multinucleated masses containing pycnotic nuclei were also noted in some tubules. The interstitial cells were present in the coagulated tissue fluid that occupied the intertubular spaces (Fig. 4). In 2 grafts a few seminiferous tubules were necrotic. Fibrous and splenic tissues invaded parts of these 2 grafts. Of particular interest was the finding of spermatids in a testis 243 days subsequent to intrasplenic transplantation. A small group of spermatids, with at least 3 cells showing late spermiogenesis, and many scattered spermatids with indication of sperm head formation were found in the seminiferous tubules. A large number of secondary spermatocytes was present in this testis (Fig. 5). In all other testicular transplants, however, spermatogenesis usually stopped at the primary spermatocyte stage.

The prostates and seminal vesicles of the castrated male mice with intrasplenic testicular transplants without adhesions were atrophic. In one mouse with vascularized adhesion of the graft to the left kidney the accessory genital organs appeared normal when examined 99 days after transplantation, and the adrenal glands showed degeneration of the x-zone. Effects of castration were observed in the Bowman's capsules of the kidneys,[9,10] and in the submaxillary glands.[11,12]

Among 18 castrated female mice with intrasplenic transplants of testes, 12 testicular transplants were recovered after a period ranging from 23 to 224 days. In 2 testes that had been grafted for 23 and 38 days, spermatogenesis had not proceeded further than the primary spermatocyte stage. Normal numbers of the interstitial cells were noted in these 2 grafts. Histological features of the testes grafted for a period of over 150 days were generally similar to the corresponding transplants in the castrated male mice, except that the interstitial cells were perhaps more numerous (Fig. 6).

The uteri of the castrated female mice bearing intrasplenic testicular grafts were atrophic and weighed less than 25 mg. No x-zone was observed in the adrenal glands; androgenic effects were not noted in the kidneys and the submaxillary glands showed castration changes.

Discussion. In view of the relatively high percentage of successful transplants, the spleen appears to be a favorable site for experiments on the intraperitoneal transplantation of testes. Pfeiffer,[3] and Turner[13] reported that testicular transplants in the livers and kidneys of rats start to develop well but usually become infiltrated with fat and connective tissue and then degenerate.

Microscopical examination of the present material reveals that the intrasplenic transplants of testes in castrated male and female mice resemble, in general, the cryptorchid

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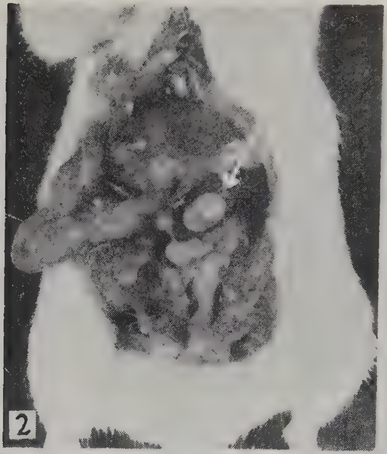

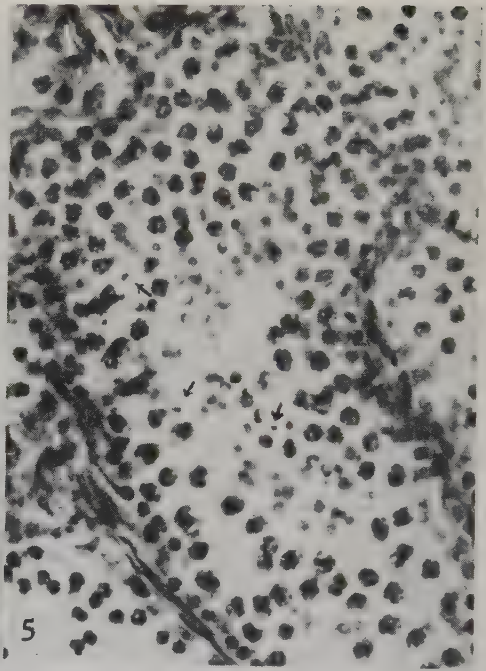

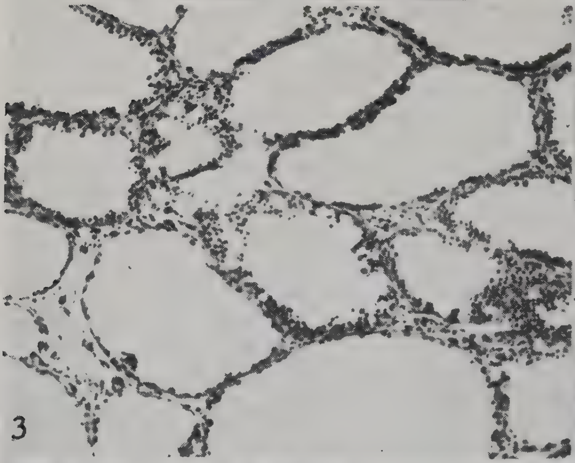

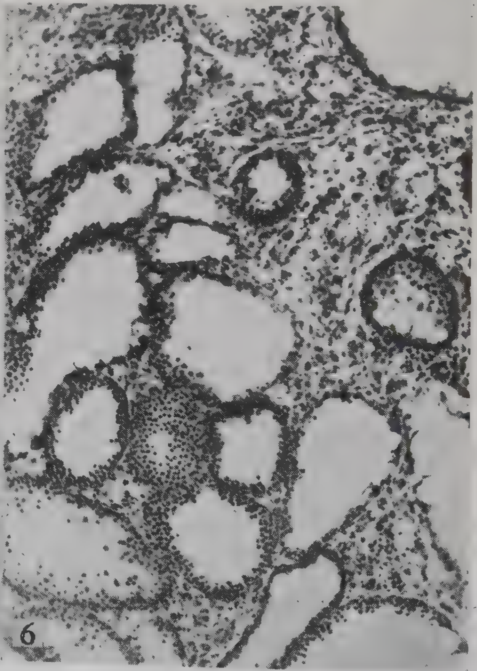

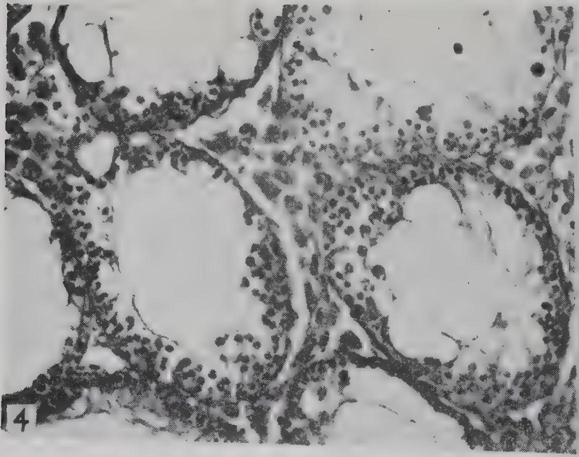

FIG. 2. Photograph of a castrated male mouse bearing an intrasplenic testicular graft for 274 days. Arrow indicates the graft. Note atrophy of the seminal vesicles and prostates.

FIG. 3. Section of a testis grafted in the spleen of a castrated male mouse for 257 days. Photomicrograph × 80 (approx.).

FIG. 4. Section of a testis grafted in the spleen of a castrated male mouse for 208 days showing the interstitial cells and disorganized seminiferous epithelium. Photomicrograph × 192 (approx.).

FIG. 5. Section of a testis grafted in the spleen of a castrated male mouse for 243 days showing spermatocytes, spermatids, and three spermiogenic cells (arrows pointed). Photomicrograph × 380 (approx.).

FIG. 6. Section of a testis grafted in the spleen of a castrated female mouse for 165 days. Note the number of the interstitial cells and thin seminiferous epithelium. Photomicrograph ×86 (approx.).

testes described in many other mammalian species.[14] That the normal spermatogenic activity in retained testes is impaired by the higher temperature in the abdominal cavity is known, but information concerning the definite mechanisms that govern the normal process of spermatogenesis is still incomplete. The observation of large primary spermatocytes containing 2 or more nuclei in the testicular grafts indicates that the cytoplasmic divisions do not proceed normally even in the spermatogonia. It is interesting to find secondary spermatocytes, spermatids, and early formation of spermatozoa in a testis transplanted into the spleen for a period of 243 days. Complete spermatogenesis in testes that presumably resided inside the abdominal cavity has been reported in 2 hermaphroditic mice by Blotevogel,[15] and Hooker and Strong.[16]

The maintenance of accessory genital organs in a castrated male mouse with vascularized adhesion of the testicular graft to the left kidney indicates that intrasplenic grafts produce androgen. The histological appearance of the interstitial cells of Leydig in the testicular transplants affords further indication of the functional activity. The castration effects found in organs of the castrated male mice bearing intrasplenic transplants of the testes suggest that the liver inactivates the androgenic hormone produced by the graft. With the mice thus physiologically castrated as far as androgen in the general circulation is concerned, it seems probable that the gonadotrophic hormones of the hypophysis are increased.[17] This would explain the functional appearance of the interstitial cells in the intrasplenic grafts and would agree with the observations of Nelson[18] and Moore[19] who reported that the hormone secretion by experimental cryptorchid testes in rats is stimulated by injections of gonadotrophic hormones. Turner[13] observed hypertrophy and hyperplasia of the interstitial cells of Leydig in intraocular transplants of testes in castrated rats, but the transplants in noncastrated rats invariably contain sparse interstitial elements.

Histological study of the present material shows no indication that the intrasplenic testicular transplants in castrated male and female mice may develop into a granulosa cell-like tumor such as reported by Biskind and Biskind[8] in a castrated male rat under similar circumstances. The failure to find tumors in the present experiment suggests that intrasplenic testicular transplants do not as readily become tumorous as do intrasplenic ovarian grafts in castrated mice.[1,2]

Summary. Thirty-two successful testicular homotransplants were recovered from 40 castrated male and female mice of the A strain bearing the intrasplenic transplants for a period ranging from 23 to 275 days. The testicular transplants showed the general histological features of cryptorchid testes, although spermatids and sperm heads were noted in one case after 243 days of transplantation. The present material gave no indication of tumor development in the intrasplenic testicular transplants. The experimental data suggested that the endogenous androgen produced by the intrasplenic testicular transplant was inactivated when passing through the hepatic portal system.

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15781

Toxicity Studies on Rutin.

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Rutin is a flavonol glycoside that has been found clinically to have a beneficial effect in a variety of conditions characterized by abnormal fragility of the capillaries.[1–4] Its action is thus similar to that of vitamin P, the term applied by Rusznyák and Szent-Györgyi[5] to a preparation which they believed led to a decrease in capillary fragility. The substances which have been reported to possess this activity have been flavonol derivatives or compounds closely related to the flavonols,[1,6] and of these rutin (quercetin rhamnoglucoside) is found in a number of common plants. The preparation of rutin from buckwheat is economically feasible.[7]

Toxicity data on the flavonol glycosides are scanty and knowledge of chronic toxicity is especially limited. Since clinical experience indicates that treatment, to be effective, must be continued for long periods, chronic toxicity studies are especially important. Garino[8] administered rutin, quercitrin, hesperidin and naringin by mouth or parenterally into dogs; some of these compounds were eliminated in the urine unchanged, and none was found to be toxic. Czimmer[9] reported, without details, that a quercetin glycoside obtained from forsythia could be administered parenterally in relatively large doses for a long period of time to a variety of animals without eliciting any symptoms. (This glycoside from forsythia has been identified by Couch, Naghski and Porter[10] as rutin). von Jeney, Vályi-Nagy, Váczi and Mihalik[11] injected 10 mg kg of quercitrin into dogs without untoward effects, although blood lactic acid values were lowered. Wilson and DeEds[12] fed diets containing up to 1% of the flavonone glycosides, naringin and hesperidin, to rats for 200 days. Growth and organ weights of the animals were not affected and the tissues of hesperidin-treated animals, which were examined microscopically, were normal. Giltner[13] has fed rutin to albino guinea pigs and exposed the animals to light; these animals did not show signs of light sensitization such as occurs in animals eating buckwheat, an important point, since the principal source of rutin at the present time is buckwheat. Clinical reports by Griffith, Couch, Lindauer[2] and by Shanno[3] indicate that a daily oral intake of 40 to 120 mg of rutin for periods up to 9 months can be taken without the patients showing any signs of toxicity from the drug, and Griffith, Lindauer, Shanno and Couch, in their exhibit at the meeting of the American Medical Association in 1946, stated that in some cases the time had been extended to 36 months.

Experimental.—Acute toxicity. In the

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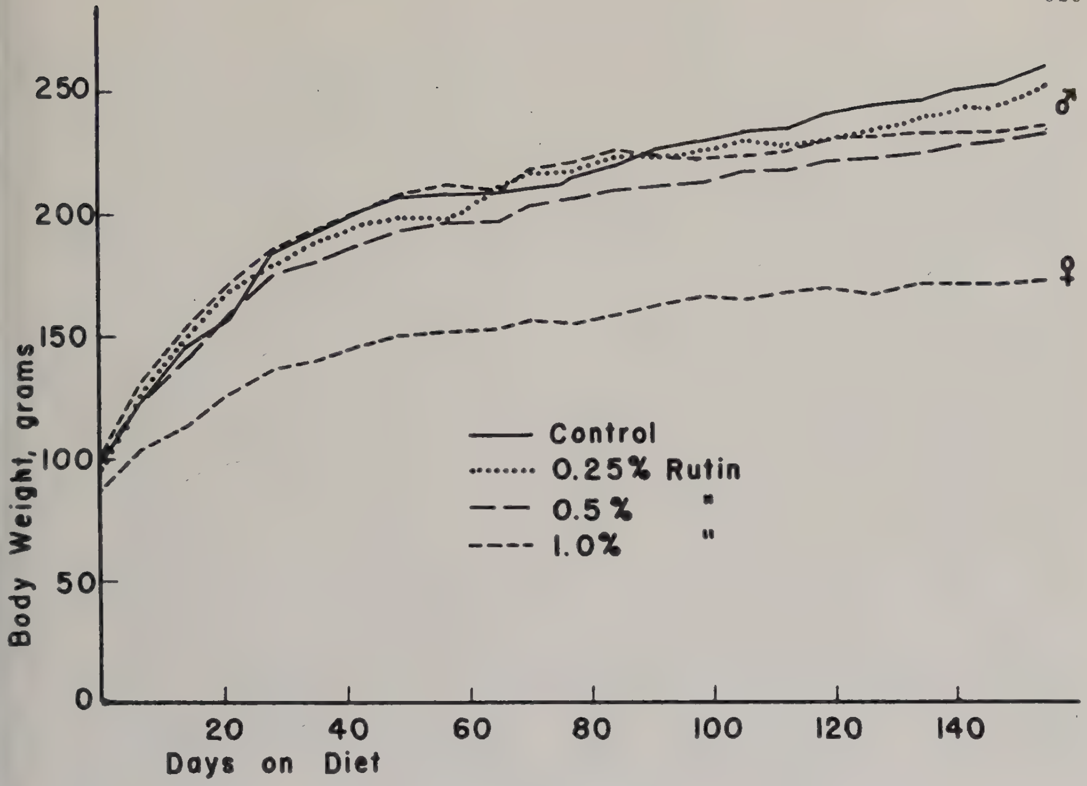

FIG. 1.
Growth curves of rats on Rutin-containing diets.

course of studies to be published later on the pharmacology of rutin,* certain data have accumulated on the acute toxicity of the substance. Adult rats and guinea pigs weighing 200 to 350 g have been injected with solutions containing 10 mg/ml of rutin. Ordinarily, the solvent was 5% ethyl alcohol, the rutin being soluble in the hot solvent and precipitating out slowly enough on cooling so that injections could be made. Occasionally, Ringer's solution was used as the solvent; rutin is fairly soluble in Ringer's, but a darkening of the solution indicated some chemical change and, for this reason, such a solution was seldom used. Sixteen rats were given 10 mg per animal, or approximately 50 mg/kg, intraperitoneally, and 8 guinea pigs 10 mg per animal, about 30 to 40 mg/kg, intraperitoneally or intravenously. Twelve rabbits were injected intravenously with a 20% solution of rutin dissolved in propylene glycol; the dosage was 100 or 200 mg/kg. No symptoms were observed in any of these animals.

Chronic toxicity. Albino rats were used for the studies of chronic toxicity. These animals, mostly males and weighing about 90 g at the start of the experiment, were divided into groups of 6 animals each and fed diets containing from 0.25 to 1.0% of rutin thoroughly mixed into finely ground Purina Dog Chow.† Growth curves and food intakes were recorded for 150 days (Fig. 1). Growth was essentially normal at all levels of rutin. The female animals receiving the 1.0% diet had no controls. These animals were used later in connection with studies on reproduction. Their growth curve is included on the chart to indicate that administration of the diet was for the same period as for the males on the same diet. At the end of this period, the control rats and those on the 0.25 and 0.5% rutin diets were autopsied and the liver, spleen, kidneys, adrenals, testes and heart were weighed. The weights of the organs did not deviate from control values to a statistically significant degree, and the gross appearance of all tissues was normal.

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* The rutin was furnished us by the Eastern Regional Research Laboratory of the U. S. Department of Agriculture, Philadelphia.

† "Purina Dog Chow" is named as part of the specification of the exact experimental conditions, and it is not implied that this product is better than or inferior to any other commercially prepared dog feed.

The 6 males and 6 females on the 1% rutin diet were continued on the diet following completion of the growth records. They were mated, with results recorded below, and afterwards observed periodically until they had been eating the diet for 400 days. At autopsy, the gross appearance of the tissues was normal; organ weights were not obtained.

Tissues from all of the animals (both the 150-day and 400-day rats) were embedded in paraffin and sections were stained with hematoxylin and eosin. Histopathological study of these sections was made by Dr. A. J. Cox, Jr., Stanford University School of Medicine, San Francisco, who reported that there were no striking changes in any of the rats as compared with the controls. The animals which received 1% rutin in the diet for 400 days showed slight irregularity in vacuolation of the cortical cells of the adrenals, which may signify a little irregularity in lipoid content of the cells. However, this was not pronounced and is of doubtful significance. In 3 of the 6 female rats which received the 1% rutin diet there was distinctly more brown granular pigment in the renal epithelial cells than in the controls. However, since all of the control animals were males, this observation cannot be interpreted. The male rats receiving the same amount of rutin did not show this change.

It was mentioned above that at the end of 150 days, the animals on the 1% rutin diet were used for observations on reproduction. There were 6 pairs, caged together for a month. From these unions there resulted 4 litters from 3 of the females (one of which must have been impregnated immediately after the birth of the first litter). One of the 4 litters was born dead and the other 3 were small (3 to 5 per litter). Since the average for the colony is considerably better than this, the experiment was repeated with variations using more animals.

At 3 months of age, 30 healthy female rats and 15 males were placed on their respective diets—Purina Dog Chow containing 1% of rutin for the experimental animals, and the chow alone for the controls. Fifteen females and all of the males ate the rutin diet. After a month the animals were divided into 15 groups, each group containing a male, a female on the rutin diet, and a control female which was a litter-mate of the experimental female. To keep the experimental animals from eating control diet, and the control rats from ingesting rutin, food was withheld during the periods when the animals of a group were caged together. These periods were limited to about 7 hours, and there were 18 of them in the month of the experiment. Admittedly such excessive handling would not be conducive to good breeding, but chances for impregnation should be equal for control and experimental rats. As a result of these matings there were 4 litters born to the females eating the rutin diet, and 2 litters to control females. The litters varied considerably in size, mortality was not extreme. It is concluded that 1% of rutin in the diet did not limit the fertility of these animals.

Several of these litters were allowed to grow until weaning. The young of the mothers on the rutin diet were as large and active as those of the control females.

As a further check on the influence of rutin on reproduction, estrus studies were made for a 4-week period on 6 female rats eating a 1% rutin diet and 6 control females. The length of the estrous cycle was not modified by the ingestion of rutin.

Discussion. Clinical use of rutin, as noted earlier, can be expected to be for long periods of time, as present indications are that discontinuance leads to a recurrence of the original condition. For this reason, the most important part of a toxicity study of rutin must be the effect of chronic ingestion. Ingestion by rats of a diet containing 1% of rutin for 400 days amounts, in the adult animal, to about 0.6 g/kg/day, and 400 days is well over one-third of a normal rat life. Such an intake is far in excess of any dosage likely to be administered for therapeutic purposes.

Conclusion and Summary. Rutin, a flavonol glycoside with vitamin P activity, was studied for signs of acute and chronic toxicity. In rats and guinea pigs, intravenous and intraperitoneal injections of 30 to 50 mg/kg, and intravenous injections in rabbits of 100 to 200 mg/kg, were inocuous. The rate of growth of albino rats was not affected when the diet contained as much as 1% of rutin, and after 400 days on such a diet, histological examination of the tissues showed no evidence of injury which could be definitely related to rutin administration. Organ weights of the experimental animals were normal. The length of the estrous cycle was the same in rats eating a 1% rutin diet as in control animals, reproduction was as good, and the young appeared to be as healthy. On the basis of the criteria employed, rutin is nontoxic both acutely and chronically.

15782

Two Antibiotics (Lavendulin and Actinorubin) Produced by Two Strains of Actinomyces. III. Toxicity and Therapeutic Studies.*

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Two new antibiotics, lavendulin and actinorubin, produced by 2 strains of Actinomyces isolated from soil have been described.[1,2] The active substances have been purified and isolated and found to be of a basic nature.[3] It is the purpose of this publication to describe the toxicity and therapeutic studies which were carried out in mice.

Materials. Test animal. White mice weighing 17-19 g were used throughout the experiments.

Antibiotics. Partially purified preparations[3] of lavendulin and actinorubin were used. The purest preparation of lavendulin used contained 4.5% ash. By the agar plate method of assay[3] 0.65 μg per ml of nutrient agar inhibited the growth of *Escherichia coli.* By the method of assay in which serial dilutions of the material are made in nutrient broth,[2] 0.078 μg per ml of broth inhibited the growth of *E. coli,* P216. The purest preparation of actinorubin used contained 2.8% ash. By the agar plate method of assay,[3] 0.55 μg per ml of nutrient agar inhibited the growth of *E. coli.* By the method of assay in which serial dilutions of the material are made in nutrient broth,[2] about 0.2 μg per ml of broth inhibited the growth of *E. coli.* It was more difficult to get a reproducible end-point in the assay of actinorubin in broth than was the case with lavendulin.

Test organism for protection studies. Klebsiella pneumoniae, American Type Culture Collection No. 8050 was used. It was found to be of serological type A by the Quellung reaction.

Technics. Dilutions of the antibiotics were prepared in sterile distilled water.

For determining toxicity a given dose of the antibiotic was injected intraperitoneally into each of 10 mice. The mice were observed daily for 14 days.

In each protection test the virulence of the

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* This project has been supported by the Smith, Kline and French Laboratories, Philadelphia, Pa.

[1] Kelner, A., Kocholaty, W., Junowicz-Kocholaty, R., and Morton, H. E., *J. Bact.*, 1946, **51**, 591.

[2] Kelner, A., and Morton, H. E., to be published.

[3] Junowicz-Kocholaty, R., and Kocholaty, W., to be published.

culture was checked. One ml amounts of an 18-hour-old broth culture of *K. pneumoniae,* serially diluted in broth, were injected intraperitoneally into mice. Usually 5 to 10 mice were injected with each dilution of the culture. At the time each dilution of the culture was injected into mice, 1 ml portions were made into poured agar plates. After 48 hours of incubation at 37°C the colonies were counted. Five to 10 bacilli, as judged by the poured agar plate count, killed over 50% of the mice, whereas 40 to 50 organisms could be depended upon to kill all of the mice. The challenging dose of culture was between 100 and 1000 times the minimum amount of culture required to kill all of the mice. For determining the therapeutic effect of the antibiotic, 100 to 1000 lethal doses of *K. pneumoniae* were injected intraperitoneally into each mouse, followed in a few seconds by an intraperitoneal injection of the drug. Usually 10 mice were injected with each graded dose of the drug. The mice were kept under observation for 14 days. The heart's blood of some of the animals dying during the course of the experiment was streaked on agar plates to determine that death was due to infection with *K. pneumoniae.*

Results. The results of injecting mice with varying dilutions of lavendulin are summarized in Table I. Intraperitoneal injection of 0.5 mg was fatal to all of 10 mice. The results of the therapeutic action of lavendulin in mice against at least 100 lethal doses of *K. pneumoniae* are summarized in Table II.

TABLE I.
Results of Injecting Mice with Varying Doses of Lavendulin.

No. of mice	Dose of lavendulin	Results
10	1 mg	1 died, < 46 hr 1 " 53 " 7 " < 70 " 1 " 70 "
10	0.75 "	1 " 50 " 6 " < 70 " 1 " < 72 " 2 " <104 "
10	0.5 "	6 " < 70 " 2 " <104 " 1 " <105 " 1 " 125 "
10	0.25 "	1 " < 71 " 3 " <104 " 1 " on 6th day 5 alive after 14 days*
10	0.1 "	10 " " 14 " †
10	75 μg	10 " " 14 " ‡
10	50 "	10 " " 14 " §

* Wt at time of injection, 17-18 g. Avg wt at end of exper., 16.9 g.

† Wt at time of injection, 17-18 g. Avg wt at end of exper., 19.3 g.

‡ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.6 g.

§ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.4 g.

TABLE II.
Summary of Tests Demonstrating the Protective Action of Lavendulin Against *K. pneumoniae* in White Mice.

Dose of culture, ml	diln	Dose of lavendulin	No. of mice injected	No. of mice dying	No. of mice surviving
1	10^{-8}	0	5	3	2
1	10^{-7}	0	5	5	0
1	10^{-6}	0	5	5	0
1	10^{-5}	0	5	5	0
1	10^{-5}	200 μg	10	0	10
1	10^{-5}	100 "	10	0	10
1	10^{-5}	50 "	10	0	10
1	10^{-5}	25 "	10	1*	9
1	10^{-5}	10 "	10	2†	8
1	10^{-5}	5 "	10	7‡	3

* Died on the 12th day of the experiment. No *K. pneumoniae* in culture from the heart's blood.

† Died on the 2nd and 3rd days of the experiment. One mouse autopsied and *K. pneumoniae* cultivated from the heart's blood.

‡ *K. pneumoniae* cultivated from the heart's blood of each of 3 mice autopsied.

TABLE III.
Results of Two Experiments in Which Mice Were Injected Intraperitoneally with Varying Doses of Actinorubin.

No. of mice	Dose of actinorubin	Results
	mg	
10	3.43	10 died (3rd and 4th days)
10	1.37	10 died (3rd, 4th, and 5th days)
10	1.0	9 died (4th, 5th, and 9th days) 1 alive 14th day
10	0.68	5 died (5th, 6th, 7th, and 10th days) 5 alive 14th day
9	0.34	9 alive 14th day
10	1	8 died on 5th day 1 died on 7th day 1 alive after 14 days*
10	0.75	3 died on 5th day 7 alive after 14 days†
10	0.5	3 died (8 to 13 days) 7 alive after 14 days‡
10	0.35	2 died, 3rd and 8th day 8 alive after 14 days§
10	0.25	All alive after 14 days§

* Wt at time of injection, 18-19 g. Wt at end of exper., 15 g.

† Wt at time of injection, 18-19 g. Avg wt of 5 mice at end of exper., 15.1 g.

‡ Wt at time of injection, 17-18 g. Avg wt at end of exper., 16.8 g.

§ Wt at time of injection, 17-18 g. Avg wt at end of exper., 19.9 g.

All of 10 mice were protected from death due to the test organism by 25 µg of lavendulin. The ratio of the therapeutic dose to toxic dose of lavendulin is of the order of 1:20. Calculating the LD_{50} of lavendulin and that dose of lavendulin which protected 50% of the mice in the presence of more than 1000 LD_{50} of culture by the procedure of Reed and Muench,[4] the ratio of therapeutic dose to toxic dose is of the order of 1:27.

The results of injecting mice with varying dilutions of actinorubin are summarized in Table III. All of 10 mice were killed by

TABLE IV.
Summary of the Tests Demonstrating the Protective Action of Actinorubin Against *K. pneumoniae* in White Mice.
Date of test: April 13, 1946.

Dose of culture ml	diln	Dose of actinorubin	No. of mice injected	No. of mice dying	No. of mice surviving
1	10^{-9}	0	5	1	4
1	10^{-8}	0	5	5	0
1	10^{-7}	0	5	5	0
1	10^{-6}	0	5	3	2
1	10^{-5}	0	5	5	0
1	10^{-6}	340 µg	5	0	5
1	10^{-6}	137 ”	5	0	5
1	10^{-6}	68.5 ”	5	0	5
1	10^{-6}	34 ”	5	0	5
1	10^{-6}	13.7 ”	5	0	5
1	10^{-6}	6.85 ”	5	1	4
1	10^{-6}	3.4 ”	5	2	3
		Date of Test: April 24, 1946.			
1	10^{-9}	0	10	1	9
1	10^{-8}	0	10	7	3
0.5	10^{-7}	0	10	9	1
1	10^{-7}	0	10	10	0
1	10^{-6}	0	10	10	0
1	10^{-5}	0	10	10	0
1	10^{-5}	40 µg	10	3	7
1	10^{-5}	30 ”	10	5	5
1	10^{-5}	20 ”	10	6	4
1	10^{-5}	14 ”	10	6	4
1	10^{-5}	10 ”	10	7	3
1	10^{-5}	5 ”	10	10	0
1	10^{-5}	2.5 ”	10	9	1

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[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

an intraperitoneal injection of 1.37 mg. The results of one test (on April 13) on the therapeutic action of actinorubin in mice against 100 to 1000 lethal doses of *K. pneumoniae* are summarized in Table IV. All of 5 mice were protected from death by 13.7 μg of actinorubin. The ratio of the therapeutic dose to the toxic dose of actinorubin in this experiment was of the order of 1:100. No explanation is offered of the fact that 2 out of the 3 mice, which received a dose of culture estimated to be in excess of 100 lethal doses of *K. pneumoniae,* failed to die.

Repeating the experiment in part, using 100 lethal doses of *K. pneumoniae*, gave less protection than in the previous test. Comparing the dose of 30 μg of actinorubin which protected 50% of the mice against 100 lethal doses of culture with 680 μg which killed 50% of the animals gives a ratio of 1:22. Practically the same ratio was obtained using LD_{50} as calculated by the method of Reed and Muench.[4] The effects of aging and repeated freezing and thawing of the solutions of actinorubin were not determined.

Since actinorubin appeared more active *in vivo* against *K. pneumoniae* than lavendulin, 2 preliminary tests were made to determine rate of absorption following intraperitoneal or oral administration. Each of 8 mice were injected intraperitoneally with 1.3 mg of actinorubin. This would ordinarily inhibit the growth of *E. coli in* Bacto-nutrient broth, pH 7.3, in a dilution of about 1:6875. After varying intervals of time one of the mice was anesthetized with diethyl ether and bled from the axilla according to the technic described by Kuhn.[5] Fifteen minutes after the intraperitoneal injection, the blood of a mouse inhibited *E. coli* in a dilution of 1:320; after 30 minutes, the blood of another mouse inhibited in a dilution of 1:160. After 75 minutes the blood of another mouse inhibited the test organism in a dilution of 1:10. No actinorubin was detected in a 1:10 dilution of blood samples taken from mice at 2-, 3-, 4-, 5- and 6-hour intervals.

Seven mice were each given 3.43 mg of actinorubin in 0.25 ml by stomach tube. No actinorubin was detectable in the blood when tested after ¼, ½, 1, 2, 3, 4, and 5 hours, one animal being sacrificed at each period.

Pathological Changes.[†] Mice which survived the larger test injections of lavendulin and actinorubin (Tables I and III) failed to gain weight or even lost weight during the 2 weeks of observations. Postmortem examination of 6 mice 16 days after the intraperitoneal injection of each with 0.75 mg of actinorubin revealed the following abnormalities: Body weights varied from 13.4 to 19.7 g (normal weight for the same lot and age was 31 g). The livers were irregularly distorted with a reduction in the number of lobes or fusion of the lobes to form a rather solid mass which often completely enclosed the gall bladder. Microscopic examination revealed eosinophilic, necrotic foci in some livers of the animals but in all fibrous tissue stroma was irregularly increased and the lobules often were more or less distorted. A true cirrhosis was not developed, however. The kidneys were enlarged and of a pale, dull brown color. In all of the animals the convoluted tubules were dilated and lined by flattened epithelial cells, whereas the glomeruli were lined by columnar cells. The dilated tubules in 4 animals contained dense eosinophilic casts. In each of the 6 animals the thymus was reduced more or less but in 4 of the animals little remained but reticulum and epithelial elements. The other viscera were unchanged.

Five mice which survived 0.25 mg of lavendulin were sacrificed 15 days after the intraperitoneal injection of the drug. Body weights varied from 12.9 to 19.9 g (normal weight for the same lot and age was 31 g). The livers were distorted to a lesser degree than in the group which received actinorubin although the abnormalities were of the same type. The kidneys were unchanged. The thymus in 3 of the animals appeared reduced in size but microscopic sections were essentially normal. The spleens were reduced in size and the capsule was thickened by fibrous tissue growth.

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[5] Kuhn, L. R., *Science*, 1946, **93**, 504.

[†] We are indebted to Dr. Herbert Ratcliffe, Department of Pathology, University of Pennsylvania, School of Medicine, for the examination of some of the test animals.

These observations indicated that the liver was extensively damaged by the substances injected but regenerated more or less completely by the end of 2 weeks. Actinorubin also caused tubular damage in the kidneys and largely destroyed the lymphoid elements of the thymus. Lymphoid tissues of the spleen did not appear to have been damaged. Fibrosis of the splenic capsule may be attributed to the intraperitoneal injection of the material. Although the mice which received actinorubin showed the more extensive pathological changes, it must be borne in mind that, on a weight basis, these mice received three times more drug than the mice which received lavendulin.

Summary. The toxic doses (LD_{100}) of actinorubin and lavendulin have been determined for white mice by intraperitoneal injection. In the case of lavendulin it was 0.5 mg, while in the case of actinorubin it was 1.37 mg. The ability of the antibiotics to protect mice from death following the intraperitoneal injection of 100 to 1000 M.L.D. of *K. pneumoniae* was determined. In the case of lavendulin this therapeutic dose was 25 μg and in the case of actinorubin it was found to be 13.7 μg in one experiment. In a second experiment 0.68 mg of actinorubin killed 50% of the mice while 30 μg protected 50% of the mice against 100 lethal doses of *K. pneumoniae*. Further testing of the 2 antibiotics has not been possible due to the limited supplies of the substances.

15783

Production of Increased Capillary Fragility in Rats Following Irradiation.*

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In the course of a clinical study of increased capillary fragility, it became desirable to develop a corresponding study in an experimental animal. A method for measuring the increase in fragility was needed as well as a means for producing increased fragility. Hundreds of unsuccessful experiments were conducted in rats and guinea pigs before the problem was satisfactorily solved. Only the successful method, however, will be described.

Method for measuring capillary fragility in rats. Under general anesthesia the peritoneal cavity is opened by a long midline longitudinal incision, care being taken to exert no traction on the peritoneum. The abdominal wall is carefully folded back until an artery and vein are disclosed. Then, under a good light, the peritoneum is grasped on each side of the vessels and stretched between the fingers so that the lumen of the vein is obliterated but the artery can still be seen pulsating. The tension is maintained for 2 minutes after which it is relaxed. The peritoneal area supplied by the vessels is carefully inspected and, if the fragility is increased, one or more petechia will appear distal to the point of venous obstruction, usually entirely separate from any visible vessel. Actually, in a positive case, 6 or 8 such hemorrhages usually occur. If desired, the process can be repeated in the same animal, using other vessels. No petechial hemorrhages occur in the normal animal.

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* The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Trustees of the University of Pennsylvania. More recently it has been continued as a project of the Army Research and Development Board.

† Atwater Kent Fellow in Medicine.

Method for producing increased capillary fragility in rats. A substance capable of emitting alpha radiation is introduced into the peritoneal cavity of normal rats. The substance used was commercial radon ointment, 500 E.S.U., (200 microcuries 1 cc)‡ prepared in either lanolin (15 animals) or olive oil (5 animals). Radon ointment contains small amounts of radon gas. Ninety per cent of the radiation is composed of alpha particles while the remainder consists of beta and gamma particles. Amounts used were 0.5 cc (2 animals), 1.0 cc (16 animals) and 1.5 cc (2 animals). When the olive oil preparation was used, the material was simply injected through a needle, while with the lanolin a small incision had to be made. No attempt was made to remove any of the material subsequently. Fragility was tested 1 to 8 weeks after the peritoneal injection. In every animal fragility was markedly increased.

Controls consisted of 5 animals injected with lanolin, 5 injected with olive oil, and 10 entirely untreated animals. None showed any petechia during the test.

Conclusion. Exposure of the peritoneal cavity of the rat to irradiation in excessive amount will produce an increase in capillary fragility.

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‡ Dosage as estimated by manufacturer.

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Effect of Rutin on Recovery Time from Radiation Injury in Rats.*

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Rutin, a flavonol glucoside derived from various plants including buckwheat, has been shown by Griffith, Lindauer and Couch[1] to decrease capillary fragility in man, when that fragility was originally increased. One of the effects of excessive radiation appears to be an increase in capillary fragility.[2] Therefore, an experiment was planned to discover whether rutin might have a favorable effect on radiation reaction.

Method. All animals were given radiation to one leg, 2385 r, in a single dose, using 200 KV, 15 ma., ½ mm cu. + 1 mm al filter, 26 cm skin target distance. At the same time half the animals were given a pellet containing 20 mg of rutin, implanted along the lateral aspect of the abdominal wall, and this was repeated thereafter every third day, for a period of 36 days. The remaining animals were not given rutin. At the end of the experimental period 23 rutin-treated animals and 26 untreated controls survived. Animals were examined every fourth day and the extent of the reaction noted. The observed reaction consisted of an erythema, a moderate swelling and, in some cases, ulceration.

Results. The extent of the initial reaction is not charted, as there was no significant

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* The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Trustees of the University of Pennsylvania. More recently it has been continued as a project of the Army Research and Development Board. Abbott Laboratories, Chicago, Ill., provided the Rutin, and, by a grant, aided the study.

† Atwater Kent Fellow in Medicine.

[1] Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 228.

[2] Griffith, J. Q., Jr., Anthony, E. M., Pendergrass, E. P., and Perryman, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 331.

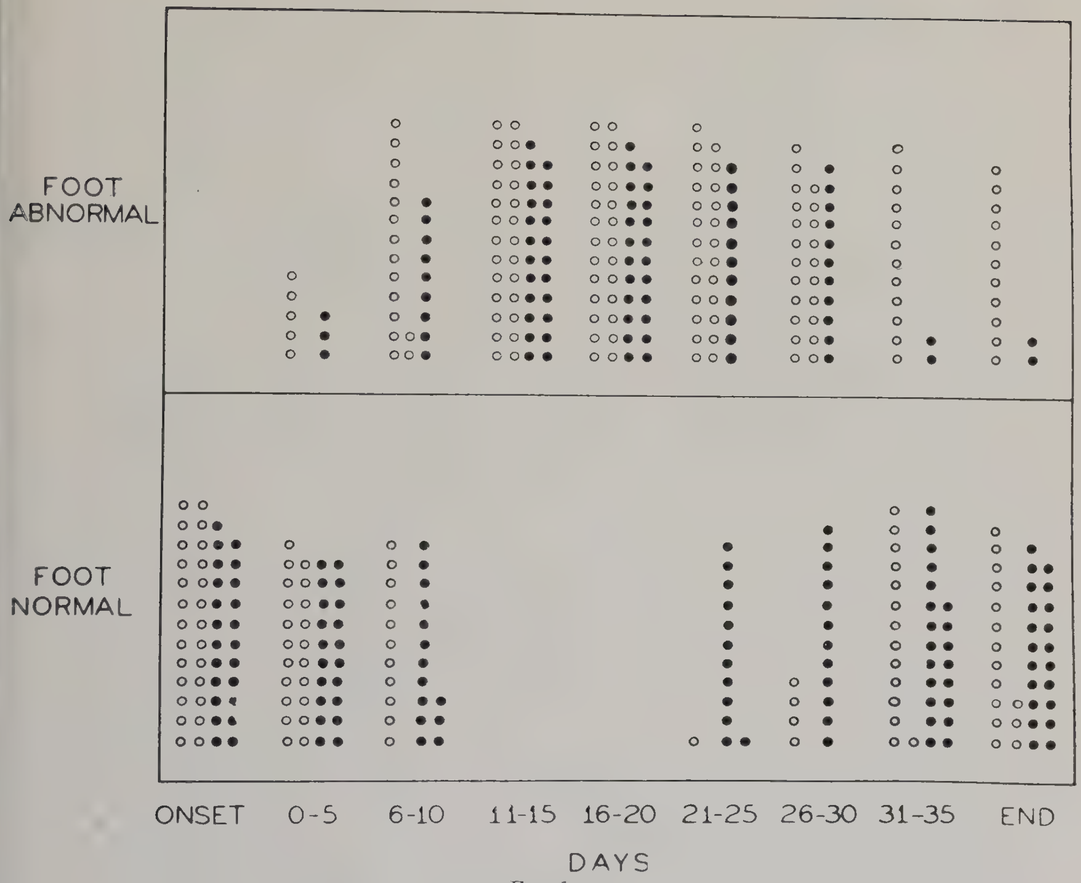

FIG. 1.

All rats were given irradiation to one foot, 2385 r in a single exposure. Closed circles represent animals treated with rutin, while open circles represent untreated animals. For other details, see text.

difference between the treated and untreated groups. There is no significant difference between the 2 groups in the time of onset of the reaction, averaging the 10th day for the control group and the 11th day for the rutin-treated group (Fig. 1). In this chart each animal is listed once for each 4-day period, appearing in the lower rectangle if the treated and untreated foot were indistinguishable, and in the upper if a definite reaction were present. It is apparent that, from the 21st to the 25th day 12 of the animals receiving rutin returned to normal and only one of the control group. At the time of termination of the experiment, after the 35th day, only 2 of the treated animals still showed an abnormal foot, while 11 of the control group were still abnormal.

Conclusion. Under the conditions of the experiment, rutin appeared to hasten the recovery time after irradiation.

15785 P

Effect of Colchicine on Resting Cells in Tissue Cultures.

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During our investigations on the effects of colchicine on cells growing *in vitro* we found that this drug, in addition to its action on the division mechanism of cells, also has a definite effect on the cytoplasm of resting cells.

The design of the experiments was as follows: Forty-eight-hour-old hanging drop cultures of chick embryo heart fibroblasts were immersed in a 10^{-4} or 10^{-5} M solution of colchicine in Ringer. The effect of colchicine was studied from the start of the experiment up to a period of 8 hours in the living state on the warm stage, and in preparations fixed in Carnoy's fluid and stained *in toto* with Giemsa's stain. In another series of experiments the colonies of fibroblasts were cultured for 24-48 hours in media containing similar concentrations of colchicine and were examined in the living state, as well as after fixation and staining.

In a culture of fibroblasts immersed in a colchicine solution of one of the above concentrations, it can be ascertained that, after 5-15 minutes the surfaces of the individual cells forming the zone of outgrowth display an extraordinary activity. The cell processes which connect neighboring cells or extend into the medium are withdrawn and the cells become independent of each other. Such a detached cell sends out pseudopodia in all directions; these pseudopodia are in constant motion, being rapidly projected and retracted. This motion is so rapid and so violent, that the peripheral portions of pseudopodia are often torn off. The cells tend to assume a polygonal or spherical shape and their cytoplasm becomes increasingly basophilic. The superficial cell layers remain active for many hours, short, broad, bleb-like pseudopodia, appearing and disappearing with great rapidity from all sides of the cell body. This picture closely resembles the behavior of the cytoplasm of dividing cells during anaphase and telophase. Often the cells become detached not singly, but as small groups of 2, 3 or more. They remain together as a small clump and this unit shows at its periphery all the phenomena described for the surface of the individual cells (Fig. 1, A and B).

The cell colony treated with colchicine soon loses its regular texture and is composed not of radially arranged spindle cells but of separated single cells and small cell conglomerates (Fig. 1, C). The process of disruption of the texture of the culture is initiated at the periphery and spreads towards the center. Usually within an hour the whole cell colony looks as if it were torn to shreds.

Most of the loosened and rounded fibroblasts do not show any signs of degeneration during the period of observation and the structure of the resting nuclei remains completely unaltered. Only occasionally there is a moderate hydropic vacuolization of the cytoplasm. Even prolonged treatment with colchicine in the above mentioned concentrations has no lethal effect on the cells. Cell colonies cultured for 48 hours in medium containing a colchicine solution 10^{-5} M show an abundant outgrowth after being transferred to normal medium.

The view has often been expressed[1-3] that the essential point in the action of colchicine on the mitotic process is the suppression of the gelation of the spindle. Our observations suggest that colchicine is able to affect not only the spindle substance but also the sol-gel equilibrium of the cortical layer in

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[1] Beams, H. W., and Evans, T. C., *Biol. Bull.*, 1940, **79**, 188.

[2] Ludford, R. J., *J. Nat. Canc. Inst.*, 1945, **6**, 89.

[3] Wilbur, K. M., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 696.

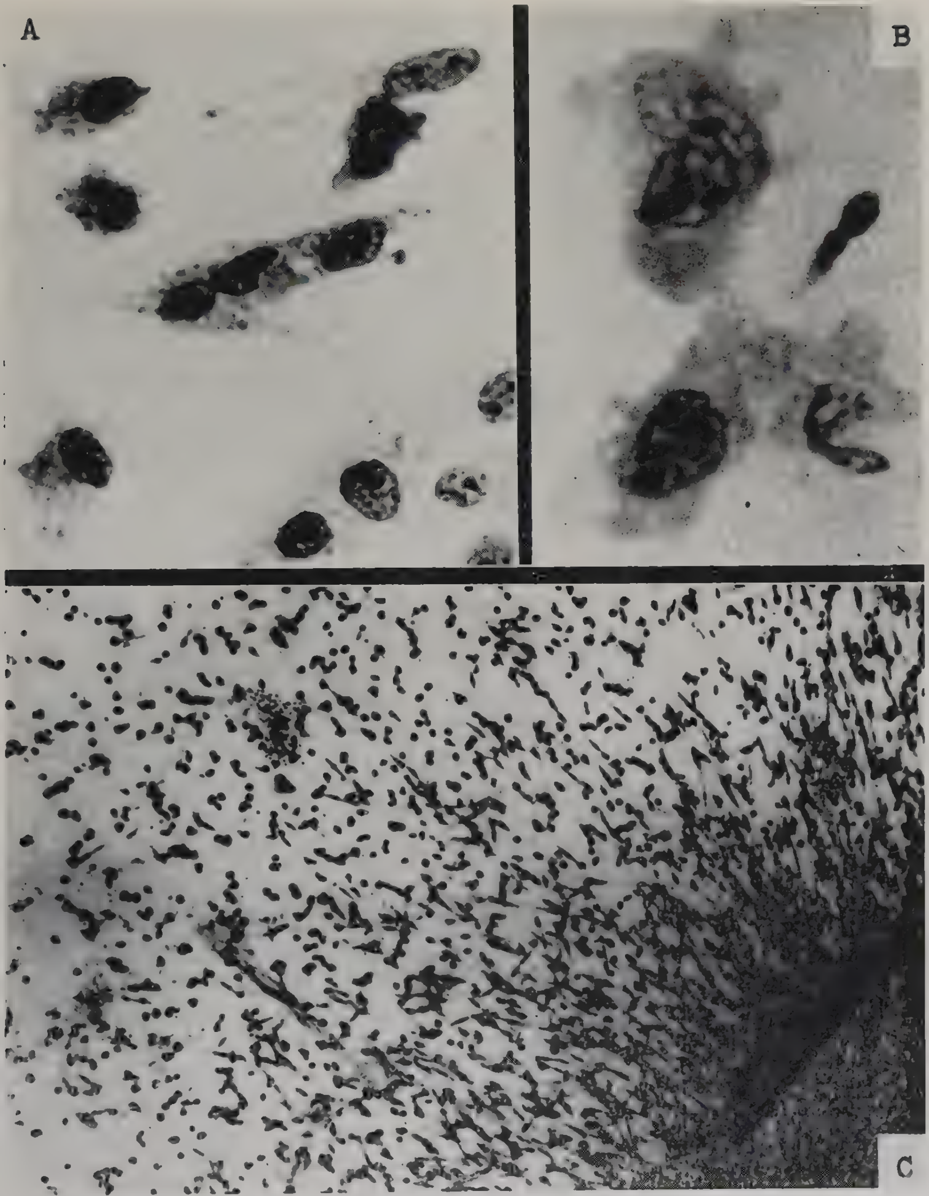

FIG. 1.
Detached and rounded fibroblasts in cultures treated with 10^{-5} M colchicine solution for periods from 15 minutes to 1 hour. Giemsa's stain. A, × 800. B, × 1800. C, × 120.

the resting cell.

As far as can be judged from the illustrations in the paper of Gavrilov and von Bistram,[4] it is possible that these authors have observed in tissue cultures treated with colchicine, the phenomenon described by us. Ludford[5] has noted that resting cells under the influence of weak solutions of colchicine tend to become less spread out and more rounded.

Summary. Chick embryo heart fibroblasts cultured *in vitro*, when immersed in colchicine solutions 10^{-4} or 10^{-5} M, show a tendency to assume a spherical shape. Their surfaces display a considerable activity as manifested by rapid protrusion and withdrawal of pseudopodia. Subsequently the zone of outgrowth of the culture splits up into detached elements, comprising one or more cells. This whole process is not lethal to the cells and is, to a great extent, reversible.

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[4] Gavrilov, W., and von Bistram, D., *Bull. Assn. franc. p. l'étude du cancer*, 1939, **28**, 319.

[5] Ludford, R. J., *Arch. f. exp. Zellforschg.*, 1936, **18**, 411.

15786

Production of Polycythemia in Rabbits by Anoxia and Cobalt.*

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A need for polycythemic rabbits as experimental animals led to the consideration of methods of producing the state of polycythemia in this species. There are at least 2 experimental methods for inducing this condition. The older and better known of these is the response to anoxia induced by high altitude; another is the administration of cobalt salts.

Although the stimulating effect of lowered oxygen tension is well known and has been extensively studied, its mechanism is not as yet clearly understood. The effect has been noted in many animals other than man.[1] These include monkeys,[2] dogs,[3] rats[3] and rabbits.[3-5]

Armstrong and Heim[4,5] reported on the exposure of young male rabbits for 4 hours daily 5 days a week to pressures equivalent to 18,000 feet. In addition to this treatment the animals were subjected to an altitude tolerance test on the 7th day. This tolerance test involved rapid decompression to the point where the animals became unconscious. The equivalent altitude attained was noted and the animal immediately returned to ground level. In the 3rd week of their experiment a deterioration set in and the hemoglobin and hematocrit levels depreciated to lower than the original values. They concluded that rabbits were quite unsatisfactory for this work.

Later Thorn, Jones, Lewis, Mitchell and Koepf[3] conducted a series of experiments in which they exposed rabbits to pressures equivalent to 18,000 and 25,000 feet for 4 hours a day 7 days a week. The 18,000-foot animals were continued for 5 weeks whereas the 25,000-foot experiment was discontinued at the end of 3 weeks because of excessive fatalities. Those exposed to 18,000 feet showed a temporary delay in growth, together with a slight drop in the concentration of serum, sodium chloride and CO_2 combining power and a decrease in the weight of the thymus gland. These changes were accompanied by an increase in O_2 capacity of the blood, in the hematocrit values and in the adrenal gland weight. Seventy-five percent of those exposed to 25,000 feet died of hemorrhage into the lungs and/or herniation of distended loops of the intestines into the thoracic cavity. There were no fatalities in the 18,000-foot group, nor was there evi-

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* This work was supported in part by the OSRD and in part by the U. S. Army Air Force, Air Material Command, Wright Field, Dayton, Ohio.

[1] McFarland, R. A., *J. Comp. Physiol.*, 1937, **23**, 244.

[2] Jasper, H. H., *Canadian National Research Council*, Ottawa, 1942.

[3] Thorn, G. W., Jones, B. F., Lewis, R. A., Mitchell, E. R., and Koeff, G. F., *Am. J. Physiol.*, 1942, **137**, 606.

[4] Armstrong, H. G., and Heim, J. W., *J. Aviation Med.*, 1938, **9**, 45.

[5] *Ibid.*, 1938, **9**, 92.

dence of degeneration in the hemopoietic system.

It may be noted that little is known of the mechanism or the optimum conditions for producing polycythemia by this method. Another technic for producing polycythemia, whose mechanism is however even less clearly understood, was reported by Waltner and Waltner.[6] They induced polycythemia by the administration of cobalt. This method has been used on several species but was found to be slow and sometimes accompanied by toxic symptoms. Frost *et al.*[7] produced a temporary polycythemia in adult dogs by including cobalt in the food. However, they found this toxic to young growing dogs. On the other hand Stanley, Hopps and Hellbaum[8] reported the development of polycythemia in rats by a subcutaneous injection without unfavorable symptoms.

Methods. It was decided to study the possibilities of both the above mentioned technics for producing experimental polycythemia. The routine of the cobalt injections was patterned after that employed by Stanley *et al.*[8] The exposure time at altitude was materially increased above the time used by previous workers, but remained intermittent. This allowed the animals some time for recuperation under more nearly optimum living conditions at sea level. Altitude and cobalt stimuli were tried separately and together on immature male rabbits. Twelve animals, averaging 3½ lb. were divided into 4 groups of 3 rabbits each. Group I served as controls remaining at sea level pressures and without cobalt injections. Group II was subjected to an ambient pressure equivalent to an altitude of 20,000 feet, 16 hours a day, 5 days a week. Group III was injected subcutaneously with 1 ml of sterile cobalt solution per pay 6 days a week, but remained at ground level (300 feet above sea level). Group IV was injected subcutaneously with 1 ml of cobalt solution per day 6 days a week and was subjected to a pressure equivalent to 20,000-foot altitude 16 hours a day 5 days a week. The cobalt solution consisted of 10 mg $CoCl_2:6H_2O$ per cc made up to isotonicity with NaCl. This was injected subcutaneously into a shaved area of the back and shoulders. The altitude was simulated in a decompression chamber. The altitude animals rested at ground levels (300 feet) each day for 8 hours and over Saturday and Sunday night, a period of 56 hours. The altitude "flights" were continued for 10 weeks. This was followed by a 2½-week ground level observation period.

The animals were weighed and sampled twice a week, on Mondays at the end of the 56-hour sea level resting period and on Thursdays after 3 of the 5 successive night flights of 16 hours. The blood samples were taken from the marginal ear vein. Hemoglobin was determined by the acid hematin method with a Klett Summerson colorimeter. Hematocrit values were measured in Wintrobe tubes. Plasma volume was determined by the dye method using T-1824.[9] The plasma volume was measured only 4 times during the course of the entire experiment. These were, first during the prestimulus period, next after 3 weeks of exposure to altitude and/or cobalt, then after 10 weeks of stimuli and lastly at the end of the poststimuli observation period. The dye determination technic consisted of the injection of a weighed amount of a 1% dye solution in isotonic saline. After 8 minutes a blood sample was taken, using 1.6% sodium oxalate solution as an anticoagulant. The blood sample-oxalate ratio was determined in each case by weighing and the ratio used to correct the readings obtained. The intensity of the blue color in the plasma was measured on a Beckman quartz spectrophotometer, using the 620 mμ absorption band.

Results. The groups of animals employed were too small to permit satisfactory statistical analysis. However, the response to the

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[6] Waltner, K., and Waltner, K., *Klin. Wchnschr.*, 1929, **8**, 313.

[7] Frost, D. V., Spitzen, E. H., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1941, **134**, 746.

[8] Stanley, A. J., Hopps, H. C., and Hellbaum, A. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 130.

[9] Price, Phillip B., and Longmeir, William P., *Johns Hopkins Hosp. Bull.*, 1942, **71**, 51.

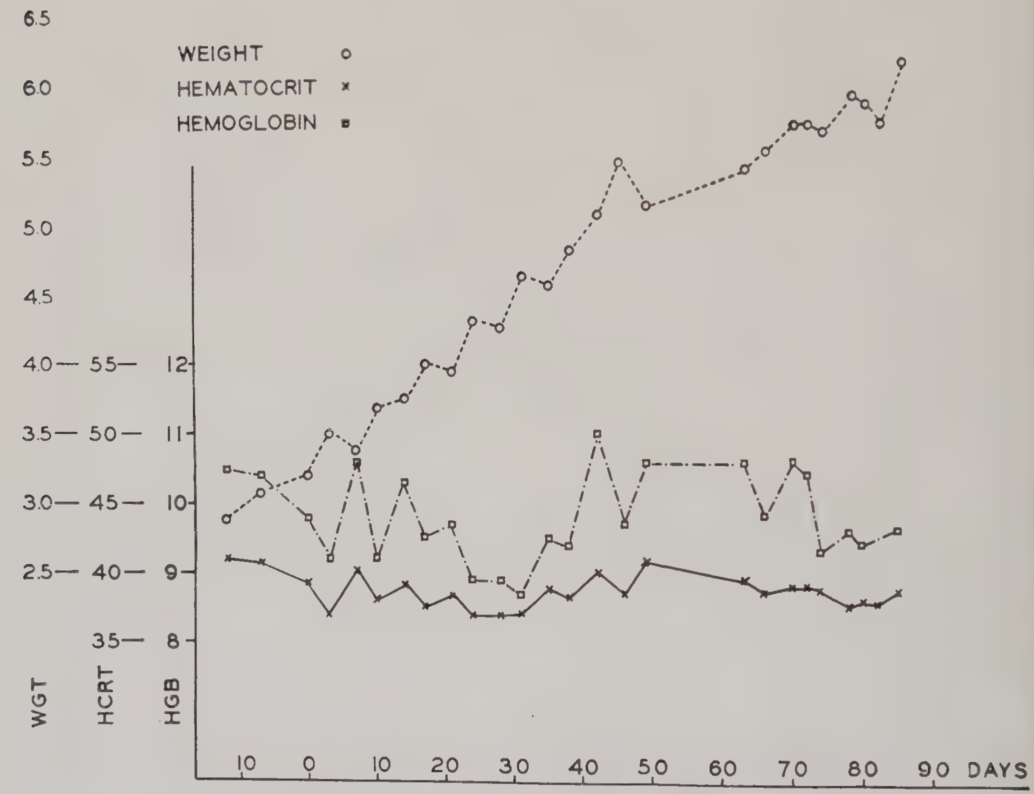

FIGURE I SEA LEVEL CONTROL

Weight, hematocrit, and hemoglobin averages of the control rabbits.

stimuli was definite and conclusively demonstrates the possibility of producing polycythemia in this species without deterioration.

Fig. 1 shows the average of the weight, hemoglobin and hematocrit values of the control group. (Only 2 animals are reported in this group. One was stolen on the second week of the experiment.)

The response to altitude alone is shown in Fig. 2. Each parameter represents the average value for 3 animals. (No fatalities). The exposure to altitude prompted a decrease in weight, followed by a period of slow recovery with a final resumption of a growth rate equivalent to that of the controls. Hemoglobin and hematocrit values both showed a rapid increase during the weekend resting period after the first exposures to altitude. This sloped off; first into a period of little change and then into a gradual climb which persisted as long as the stimulus was applied. The decrease in hemoglobin and hematocrit from 25th to 35th day coincided with a similar depression in the control group and a period of high summer heat. It is to be noted that, during the control period following the altitude exposure the hemoglobin and hematocrit values returned rapidly toward the normal levels.

One of the cobalt-injected, ground level group, died apparently from the toxic effects of the cobalt combined with severe anorexia. The data in Fig. 3 represent the average of the values obtained from the 2 remaining animals. The weight curve generally follows that of the controls. The hemoglobin value dropped slightly when the injection started, followed by a gradual increase for the period of the injections, but did not attain values comparable to those in the animals subjected to altitude. The return to normal after in-

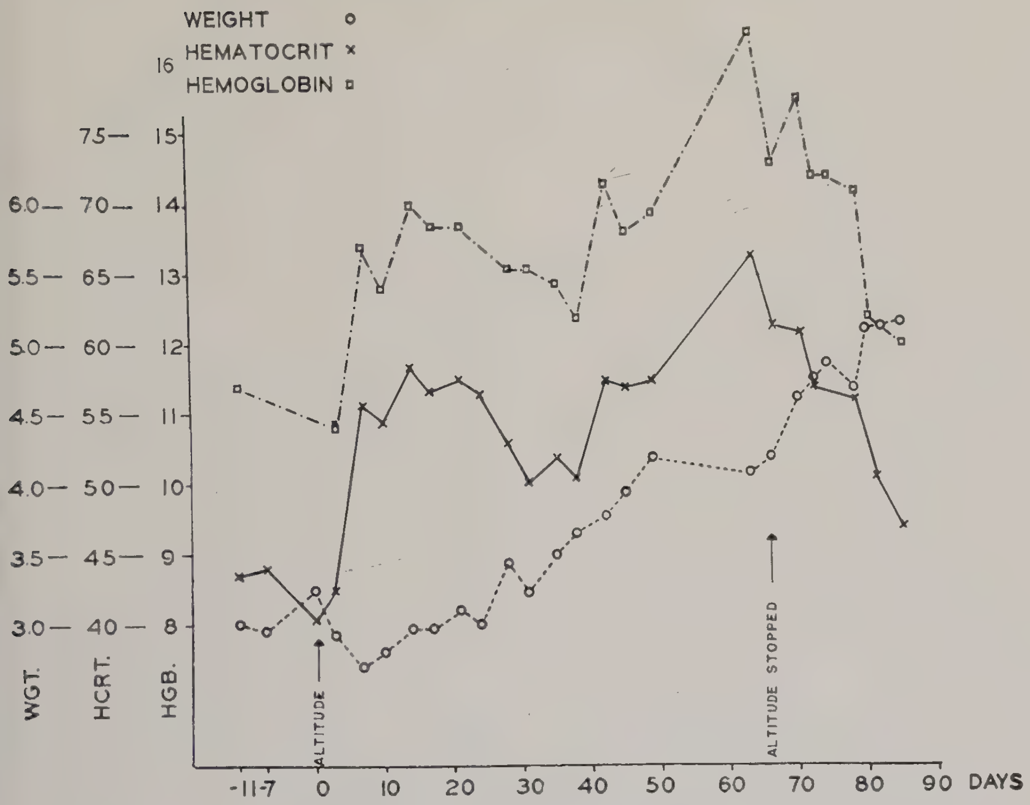

FIGURE 2 EFFECT OF ALTITUDE

Weight, hematocrit, and hemoglobin averages of rabbits having had ten weeks of intermittent altitude exposure.

jections were stopped, was slower than that found with the altitude rabbits.

Two of the 3 animals receiving combined altitude and cobalt treatment died; one apparently from the toxic effects of the cobalt, the other from the combination of toxic effects and peritonitis. Sterile abscesses developed on all 6 animals receiving cobalt injections at or near the site of the injection. The larger of these were opened and drained; however, no infections developed in these areas. The curves for the one surviving altitude and cobalt animal are shown in Fig. 4. They closely resemble those found in the Group II or altitude only animals, with the exception of the absence of the depression of hemoglobin and hematocrit values from 25th to 35th day. This may be due to individual variation. The differences are not sufficient to indicate that the 2 stimuli are additive in producing polycythemia. However, a more prolonged experiment might show this to be true.

The plasma volume and whole blood volume data are given in Table I, as the average of each group in per cent increase above the prestimulus period. In this manner each animal is compared to himself at the start of the experiment, and as all the animals were still growing all values are positive. The significance can then be shown by comparing the various groups to the control group. The whole blood volume values were obtained from the plasma volume and the corresponding hematocrit value.

The values in parentheses show the per cent change found during each period (*i.e.* the increase or decrease in percentage of each period as compared with that of the preceding period). From this one can more easily

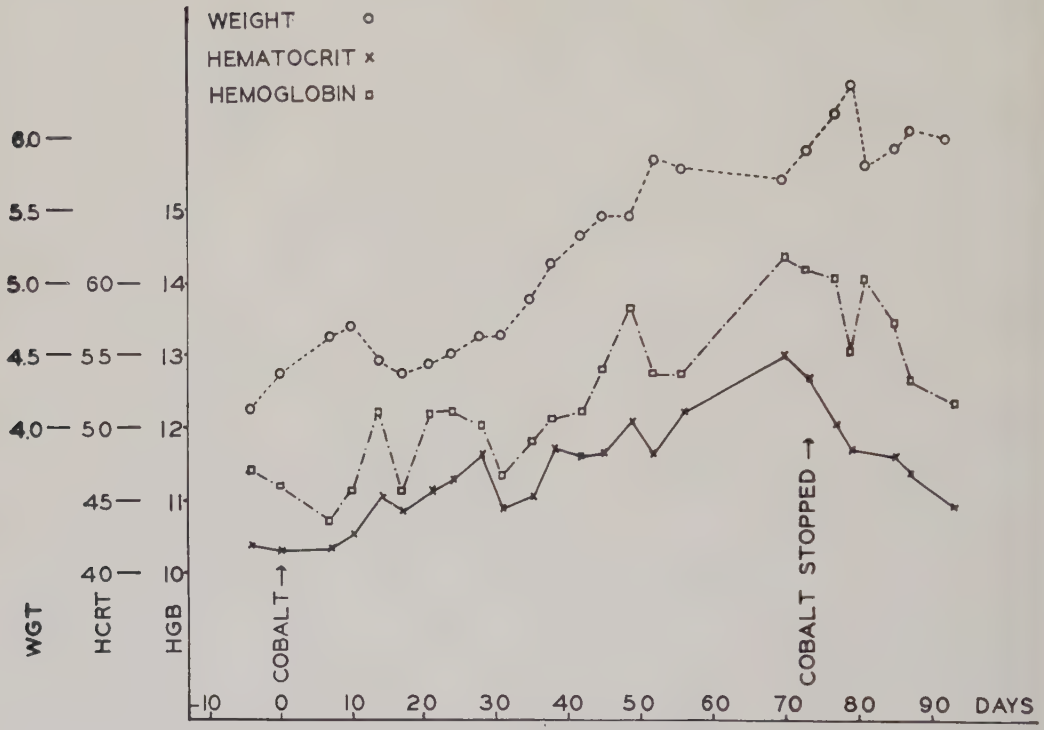

FIGURE 3 EFFECT OF COBALT

Weight, hematocrit, and hemoglobin averages of rabbits given daily cobalt injections.

follow the progressive changes at each period of the experiment.

After 3 weeks of stimuli (Table I, Column 4) there was no significant increase in whole blood volume above that of the controls. However, the hematocrits of those exposed to altitude showed an increased 45% in red cell volume at the expense of the normal increase in plasma volume. The animals exposed to altitude showed almost no change in plasma volume (3% as compared to 50% for the controls, Table I, Column 1).

During the ensuing 7 weeks of exposure to stimuli, the increase of plasma volume in the experimental animals was comparable to the increase in the controls (from 22.5% increase to 32.1% increase). At the same time the increase in the whole blood volume of the altitude animals (66% and 68%) was more than double the increase of the controls (24.2%). This suggests that the development of polycythemia may have 2 phases or processes; an initial immediate and rapid one involving an increase in the cellular phase at the expense of the plasma volume and a second more gradual step during which the plasma volume increases at a normal rate together with a further increase in the red cell volume.

The changes occurring after removal of the stimuli are for the most part in the red cell component of the blood. The plasma volume of the altitude animals increased above that of the controls and there is a large drop in the total blood volume. The behavior of the altitude plus cobalt animals was similar to that of the controls as far as the plasma volume changes were concerned but there was a large drop in whole blood volume.

Even after 10 weeks of cobalt injections Group III did not have a greater whole blood volume than that of the controls. However, they showed only a moderate polycythemia which was attained at the expense of the plasma volume. Yet after the stimulus had

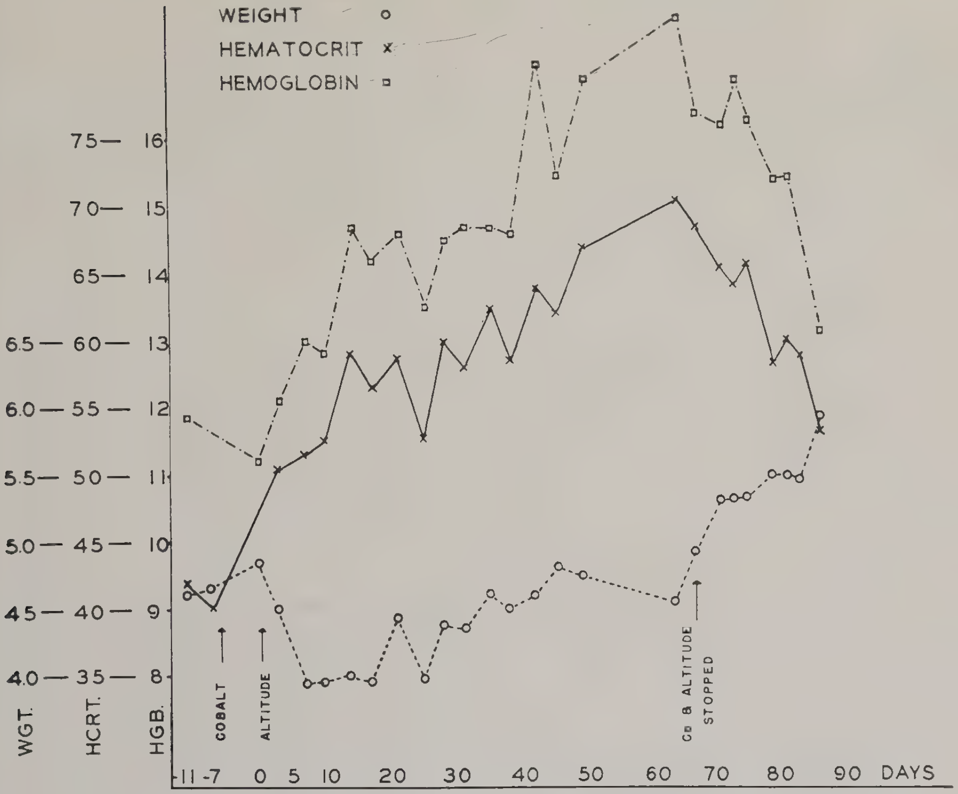

FIGURE 4 EFFECT OF COBALT AND ALTITUDE

Weight, hematocrit, and hemoglobin values of rabbit surviving ten weeks of intermittent altitude and cobalt.

TABLE I.
Percent Increase Above the Pre-experimental Value.

	Plasma volume			Whole blood volume		
	After 3 weeks of stimulus	10 wks of stimulus	2½ wks of recovery	After 3 wks of stimulus	10 wks of stimulus	2½ wks of recovery
Column No.	1	2	3	4	5	6
Control	+50.0%	+72.5%	+86.0%	+39.6%	+ 63.8%	+74.5%
		(+22.5)*	(+13.5)		(+24.2)	(+10.7)
Cobalt and altitude	+ 3.3	+28.0	+33.0	+40.4	+107.0	+60.0
		(+24.7)	(+ 5.0)		(+66.6)	(—47.0)
Altitude	+ 2.6	+34.7	+57.7	+33.7	+102.2	+69.3
		(+32.1)	(+23.0)		(+68.5)	(—32.9)
Cobalt	+12.3	+40.5	+33.5	+26.0	+ 63.3	+40.0
		(+28.2)	(— 7.0)		(+37.3)	(—23.3)

* Values in parentheses show the changes in percent increase above the values of the previous period.

been stopped, there was a decrease in plasma volume as well as total red cell volume.

This either indicates that the development of cobalt and altitude polycythemia involves different mechanisms of response, or that the cobalt animals at 10 weeks had only developed to a stage comparable to the first 3 weeks of altitude exposure.

Conclusion. Polycythemia can be rapidly induced in rabbits by exposure to altitudes of 20,000 feet 16 hours a day, 5 days a week. It would appear that there is no additive effect when cobalt injections are combined with the altitude exposure.

There may be 2 stages in the development of polycythemia. The first phase is characterized by a normal whole blood volume with a subnormal plasma volume. The second phase shows a normal rate of increase in plasma volume with a further increase in whole blood volume.

Subcutaneous cobalt injections slowly induce polycythemia in rabbits but they are accompanied by anorexia, sterile abscesses and other symptoms of toxic effects.

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Effect of Streptomycin on Growth of Rickettsiae in Eggs.

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In experimental infections, rickettsiae have been shown to be susceptible to the action of penicillin[1] as well as to other therapeutic agents.[2-6] In view of the increasing availability of streptomycin, it was of interest to determine its effect on the growth of representative pathogenic rickettsiae. Using the yolk sac technic of Cox,[7] the action of streptomycin on the growth of the rickettsiae of the Wilmington strain of murine typhus, the Breinl strain of epidemic typhus and the Karp strain of tsutsugamushi disease in embryonated eggs was studied and compared with the action of *p*-aminobenzoic acid (PABA).

Methods. Streptomycin dissolved in 0.85% saline was sterilized by filtration through a Seitz filter previously washed with distilled water. A sterile solution of PABA in buffered saline was also prepared. The concentration of these 2 solutions was so adjusted that the dosage employed was contained in 0.4 ml, which was inoculated into the yolk sac of 7-day-old embryonated eggs. The same volume of sterile buffered saline was inoculated into control eggs. Two hours later, both treated and control eggs were inoculated by the same route with a suspension of yolk sacs infected with the Breinl strain of *Rickettsia prowazeki* or the Wilmington strain of *R. mooseri* or the Karp strain of *R. orientalis*. The infective suspensions had been previously titrated in eggs; a dilution was selected which just permitted the survival of a majority of the embryos for 7 days. Uninfected control eggs received injections of the same amounts of streptomycin as a check on its toxicity. The activity of the streptomycin was checked by testing dilutions of the stock solution against a susceptible strain of *Escherichia coli*. Some of the eggs received a second injection of streptomycin 72 hours later.

The eggs were incubated at 35°C for 7 days and candled daily; the dead eggs were discarded. Deaths occurring before the 3rd day were considered to be nonspecific and were disregarded. On the 7th day of incubation, the yolk sacs from all survivors in each group were harvested, divided into

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[1] Greiff, D., and Pinkerton, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 116.

[2] Snyder, J. C., Maier, J., and Anderson, C. R., Report to the Division of Medical Sciences, National Research Council, Washington, D.C., Dec. 26, 1942.

[3] Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

[4] Hamilton, H. L., Plotz, H., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 255.

[5] Hamilton, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 220.

[6] Smadel, J. E., Snyder, J. C., Jackson, E. B., Fox, J. P., and Hamilton, H. L., (Abstract), *Fed. Proc.*, 1946, **5**, 254.

[7] Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

TABLE I.

Effects of Streptomycin and *p*-Aminobenzoic Acid on the Multiplication of *Rickettsia prowazeki*, *R. mooseri*, and *R. orientalis* in Embryonated Hens' Eggs.

Strain	Experiment	Drug	Dose for each egg, mg	Interval between therapy and infection, hr	Ratio surviving embryos to total in groups at 7th day	No. of viable rickettsiae per ml of yolk sac pools*
R. prowazeki (Breinl strain)	I	Streptomycin	0.5	−2	13/15	$10^{6.3}$, $10^{5.3}$
		,,	0.5	−2, +72	11/15	$10^{7.9}$, $>10^{6.3}$
		PABA	5.5	−2	11/14	$<10^{1.3}$
		Saline		−2, +72	7/9	$10^{8.3}$, $10^{9.0}$
		Drug controls				
		Streptomycin	0.5	−2, +72	11/13	
	II	Streptomycin	0.5	−2	7/9	$>10^{6.3}$
		,,	1.0	−2	11/12	$10^{7.8}$, $>10^{6.3}$
		,,	2.0	−2	14/15	$10^{7.6}$, $>10^{6.3}$
		Saline		−2	13/15	$10^{8.6}$
	III	Streptomycin	2.0	−2	9/12	$10^{5.9}$, $10^{7.3}$
		Saline		−2	9/12	$10^{8.4}$, $10^{8.8}$
R. mooseri (Wilmington strain)	IV	Streptomycin	2.0	−2	11/13	$10^{6.2}$, $10^{6.3}$
		PABA	11.0	−2	4/8	$<10^{1.3}$
		Saline		−2	13/14	$10^{7.6}$, $10^{7.3}$
		Drug controls	2.0	−2	9/10	
R. orientalis (Karp strain)	V	Streptomycin	2.0	−2	11/13	$10^{5.3}$, $10^{5.0}$
		PABA	11.0	−2	4/8	$10^{1.7}$
		Saline		−2	12/13	$10^{5.8}$, $10^{5.6}$

* Numbers estimated from 50% end points in animal tests. See text for description of methods. When 2 figures are given, they represent values from duplicate determinations. The symbols > and < indicate that the end points fell beyond the titration dilutions employed.

2 lots each containing 3 to 7 sacs, and shaken in bottles with glass beads and an appropriate amount of sterile diluent (nutrient broth for *R. prowazeki* and *R. mooseri* and skim milk for *R. orientalis*) to make a 50% suspension and stored in sealed glass ampoules in a carbon dioxide cabinet.

Subsequently these yolk sac suspensions were thawed, lightly centrifuged, and diluted in broth or skim milk for injection into animals. The serial 10-fold dilutions of yolk sacs infected with the Breinl strain of *R. prowazeki* were inoculated intraperitoneally into 4 cotton rats for each dilution. Three weeks later, all of these rats were tested for immunity by the intracardial inoculation of approximately 4 "certainly fatal doses" of yolk sacs infected with the Breinl strain. The 50% immunizing end-point of the treated and control yolk sac pools was calculated[8] on the basis of the immunity produced in cotton rats in such challenge experiments.

The yolk sac pools infected with the Wilmington strain of *R. mooseri* were titrated by injecting 0.25 ml of the serial 10-fold dilutions in nutrient broth into each of the 8 albino mice, by the intraperitoneal route. Three weeks after injection, the surviving mice were tested for immunity by the intravenous injection of 0.2 ml of an infected yolk sac suspension containing 4 LD_{50} "toxic doses." The 50% immunizing end-points of the drug-treated and control yolk sac pools were calculated on the basis of the number of mice surviving 10 days after the challenge.

Titrations of the yolk sac pools infected with the Karp strain of *R. orientalis* were carried out by injecting 0.25 ml of the serial 10-fold dilutions prepared in skim milk intraperitoneally into albino mice using 8 mice per each dilution. The mice were observed for 21 days, and the 50% end-points were calculated on the basis of the survivors in each group of 8 mice.

For convenience in evaluating results the minimal quantity of viable rickettsiae required to produce immunity in cotton rats (*R. prowazeki* and *R. mooseri*) or death in white mice (*R. orientalis*) is assumed to be one viable organism. It is appreciated that the minimal quantity may be more than one organism, but for the purpose of comparison in these tests, this point is disregarded.

Results. The data are summarized in Table I. Streptomycin appears to have some inhibiting effect on the growth of *R. prowazeki* in embryonated eggs. The "numbers of viable rickettsiae" in eggs infected with *R. prowazeki* and injected with 0.5 mg of streptomycin were $10^{5.3}$ and $10^{6.3}$, as compared with $10^{8.3}$ and $10^{9.0}$ in the control eggs. A second injection of 0.5 mg, 72 hours after infection did not increase this effect. Increasing the amount of streptomycin to 1.0 and 2.0 mg did not appear to increase the inhibitory effect. The mean value of the numbers of *R. prowazeki* in streptomycin-treated eggs in the 3 experiments (7 titrations which properly spanned the end-points) was $10^{6.9}$ as compared with the mean value of $10^{8.6}$ attained in control eggs (5 titrations). The difference is statistically significant (Student's "t" test; P lies between 0.05 and 0.02).

In the case of *R. mooseri,* the mean value for the eggs receiving 2.0 mg of streptomycin was $10^{6.3}$ rickettsiae as compared with $10^{7.5}$ for the controls. This difference would be expected to occur by chance only once in more than 20 times.* These effects are small when compared to the striking reduction of growth of both *R. prowazeki* and *R. mooseri* with PABA.

Streptomycin in doses of 2.0 mg per egg had no significant effect on the growth of *R. orientalis* in embryonated eggs. This is demonstrated by the "numbers of viable rickettsiae" in yolk sacs from treated eggs, $10^{5.3}$ and $10^{5.0}$, as compared with $10^{5.8}$ and $10^{5.6}$ in the untreated controls. These results with 2.0 mg of streptomycin are in contrast with those obtained with 11 mg of PABA which produced a 10,000-fold decrease in the amount of growth obtained with *R. orientalis.*†

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[8] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

* The technic of titration and the reproducible nature of the results obtained with it will be discussed in detail in another publication.

Two experiments were performed to determine whether streptomycin was rapidly inactivated in the chick embryo and thus might appear to have little effect on rickettsial growth when compared with PABA (which is known to remain at adequate levels in the egg for the period of time covered by these experiments.)[9] Streptomycin does not appear to be inactivated, since allantoic fluids and yolks harvested 4 days and 7 days after the inoculation of eggs with 1.0 mg by the yolk sac route were shown to contain the expected amounts, that is, between 5 and 25 μg per ml, by assay with 3 strains of organisms susceptible to streptomycin, *E. coli* (Waksman) and *Bacillus friedländeri* (Yarsik) and *B. friedländeri* (Lundgren).

Discussion. The concentrations of streptomycin in these tests were selected to represent approximately the concentrations attainable in man. With therapeutic effects in eggs of such a slight degree, it seems unlikely that streptomycin would have any very striking effect on human infections caused by *R. prowazeki*, *R. mooseri*, or *R. orientalis*. The effects are sufficient, however, to caution against the use of streptomycin in the attempt to isolate *R. prowazeki* or *R. mooseri* from material contaminated with streptomycin-sensitive bacteria, particularly if the numbers of rickettsiae in the material are small.

Summary. Streptomycin in amounts of from 0.5 to 2.0 mg injected into infected embryonated eggs has a definite slight inhibitory action on the growth of *R. prowazeki*. In the case of *R. mooseri*, 2.0 mg of streptomycin had a small but significant effect. With *R. orientalis*, a dose of 2.0 mg per egg appeared to have no significant effect on the multiplication of the rickettsiae. By contrast, appropriate doses of PABA (5.5 mg per egg for *R. prowazeki*, and 11.0 mg per egg for *R. mooseri* and *R. orientalis*) produced a striking inhibition of growth, reducing the numbers of viable rickettsiae by 10,000 to over 10,000,000 times as compared with untreated controls.

Addendum. As this manuscript was in preparation, Dr. J. E. Smadel informed the authors that his experiments showed a very definite therapeutic effect of streptomycin against infection of embryonated hens' eggs with *R. prowazeki* when doses of 10 mg (10,000 units) per egg were employed, 5 times the maximum dose administered in the experiments herein reported.

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† The minimum concentration of PABA required to produce a detectable inhibition of the growth of *R. orientalis* is considerably greater than that required for inhibition of *R. prowazeki*.[9]

[9] Snyder, J. C., and Stevens, D. A., unpublished observations.

15788

Action of Curare on Temperature Changes in the Brain in Combination With Pentobarbital Narcosis.

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It has been demonstrated that curare-alkaloids (Merck strychnos curare, crystallized tubocurarine hydrochloride) and dihydroerythroidine bromide have a central action, besides the peripheral effect on the neuromuscular junction.[1,2] The central action of these alkaloids was first demonstrated on the electroencephalogram of frogs. At first the amplitude and the frequency of the action

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* Aided by a grant from the Ella Sachs Plotz Foundation for Advancement of Scientific Investigation.

[1] Feitelberg, S., and Pick, E. P., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 654.

[2] Pick, E. P., and Unna, K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 59.

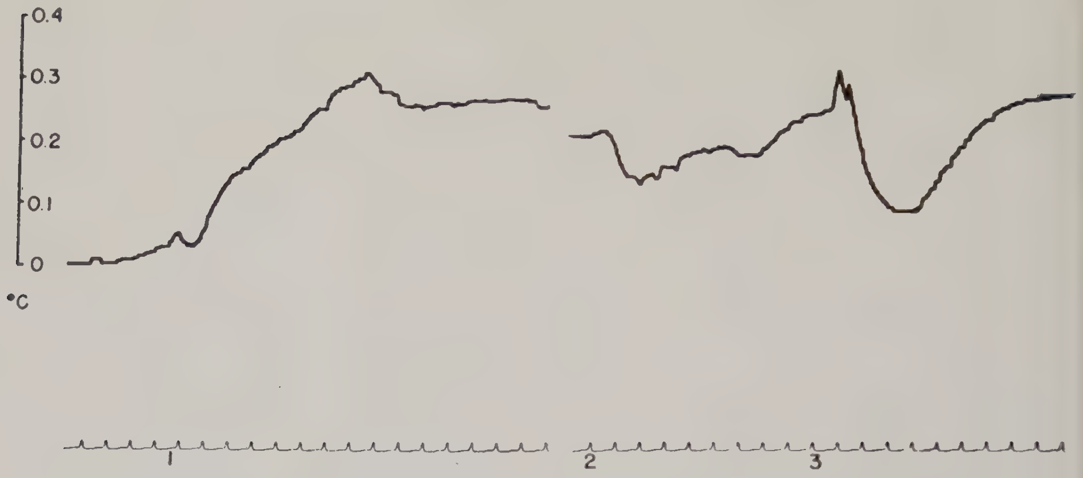

Fig. 1.

Effect of strychnos curare on temperature in the brain during light pentobarbital narcosis. Cat, 3.0 kg, narcotized with pentobarbital 15 mg/kg intraperitoneally and given artificial respiration. Time interval: 1 minute; temperature interval: 0.1°C.

(1) 2 mg strychnos *curare* intravenously causes a *rise* of 0.3°C in the brain temperature, which lasts about 8 minutes.

(2) 30 mg per kg *pentobarbital* intraperitoneally causes a slight drop of the brain temperature which returns to original level in 4 minutes.

(3) 2 mg strychnos *curare* causes a sudden drop in temperature of the brain (0.2°C) lasting about 4 minutes, which returns to normal after about 6 more minutes.

potentials are diminished. Gradually, they disappear completely. The same behavior of the electrical activity in the brain is observed in narcosis. This central action is independent of the peripheral paralysis. It seems desirable to investigate the effect of curare on the brain of mammals with the method of measurement of temperature changes in the brain, developed by Feitelberg and Lampl.[3] Since it has been shown by Pick and Richards[4] that pentobarbital and curare (and curare-like alkaloids) have a synergistic hypnotic action it is interesting to examine to what extent the temperature changes in the brain is influenced by curare in pentobarbital narcosis of varying depth.

The experiments were carried out on cats (average weight 2.5 to 3.5 kg), in pentobarbital narcosis of varying depth, under artificial respiration. Curare (Merck strychnos curare, 1.5 mg per cc of saline solution) was administered intravenously. We began the experiments with light pentobarbital narcosis (15-20 mg/kg); in other experiments we used larger doses of pentobarbital (30 mg/kg) to produce a deeper narcosis. The temperature changes were measured by a method described in detail elsewhere.[3] This method consists essentially in the following: one junction of a thermocouple is introduced into the internal carotid artery, and the other junction into the brain; the difference of temperature between the 2 junctions (between the brain and the carotid blood) is measured by a galvanometer of adequate sensitivity; this difference of temperature is interpreted as a measure of oxidation processes in the brain. The obvious objection that the difference in temperature is influenced by change in blood flow and not by temperature changes in the brain has been discussed elsewhere.[3] It suffices to mention here that the experimental findings on the behavior of temperature changes in the brain cannot be explained only by circulatory processes eventually produced by curare.

1. *Combined action of curare and light pentobarbital narcosis.* Most of our experiments on animals in light pentobarbital an-

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[3] Feitelberg, S., and Lampl, H., *Arch. f. exp. Path. und Pharm.*, 1935, **177**, 600, 725.

[4] Pick, E. P., and Richards, G. V., in print, *J. Pharm and Exp. Ther.*, 1947.

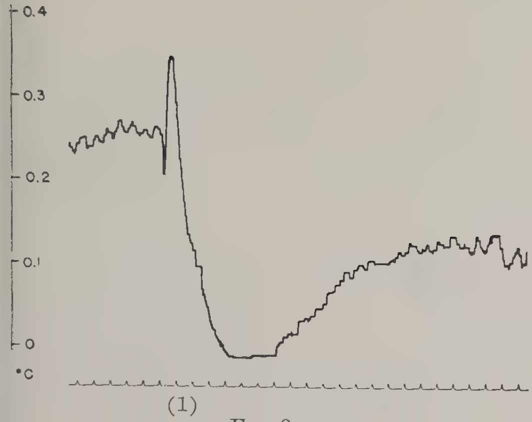

FIG. 2.
Effect of strychnos curare on temperature in the brain during deep pentobarbital narcosis and artificial respiration. Cat, 3.5 kg, narcotized with pentobarbital 30 mg/kg intraperitoneally. Time interval: 1 minute; temperature interval: 0.1°C. At (1) 3 mg strychnos *curare* intravenously causes a *sudden drop* in the brain temperature corresponding to 0.25°C, lasting about 7 minutes.

esthesia show that administration of curare causes a rise in temperature of the brain (Fig. 1). This indicates that curare, given in light narcosis without complete peripheral paralysis, has a stimulating effect on the brain. The increased temperature lasts for 10 to 15 minutes. This effect can be elicited several times on the same narcotised animal. It is independent of the peripheral body temperature. It may well be the result of actual increase in brain metabolism.

2. *Combined action of curare and deep pentobarbital narcosis.* The effect of curare is reversed if the pentobarbital narcosis is deeper, when the peripheral muscle movements are abolished by the narcosis. Under those conditions curare causes a marked decrease in temperature of the brain, lasting 5 to 15 minutes, which may be interpreted as a depression of the oxidation processes in the brain (Fig. 2). It appears therefore that a certain depth of pentobarbital narcosis is necessary for the occurrence of synergism between curare and pentobarital narcosis. No synergism seems to exist when the pentobarbital narcosis is light.

3. *Discussion.* The stimulating and depressing action of curare which we found in experiments on cerebral temperature is in agreement with the experiments reported by McIntyre *et al.*,[5] on the electroencephalogram of dogs in light pentobarbital narcosis after intravenous injection of *d*-tubocurarine. The initial effect of curare was an increase of electrical brain activity in the occipital, parietal and frontal region with a 3-fold increase in amplitude and occasional bursts of high frequency. This stage was followed shortly by a depression of electrical activity. With larger doses the depression occurred earlier and the stage of initial stimulation was brief. The latter findings agree with the earlier statements of Feitelberg and Pick[1] and Pick and Unna[2] about the depressing action of curare and curare-like alkaloids on the electroencephalogram of frogs.

The central stimulating action of curare producing hyperexcitability, accelerated respiration, central vagus stimulation and clonic convulsions was also found recently in experiments with potcurare and purified curarine chloride in cats by v. Euler and Wahlund[6] and in experiments with nonfatal doses of intocostrin and crystalline *d*-tubocurarine chloride on various mammalian species by Cohnberg;[7] central nervous depressants, *i.e.*, sodium amytal or cyclopropane decrease, abolish or prevent the stimulating central effect.

It is further interesting to note that in earlier investigations of the action of *d*-tubocurarine on frogs, Tillie[8] and Jacabhazy[9] have described a stimulation of the brain followed by a general reflex inhibition and paralysis of the spinal cord. According to Fuehner[10] guanidine, which has curare-like properties, produces also an initial central excitation in frogs, followed later by a central paralysis, which occurs 2 hours before peripheral paralysis appears.

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4. *Summary.* Curare, which affects the electrical activity of the brain has also a distinct effect on the temperature in the brain in combination with pentobarbital narcosis. These temperature changes in the brain indicating changes of oxidation processes are increased by curare under light pentobarbital narcosis, and decreased under deep pentobarbital narcosis. This latter central action may be involved in the synergistic narcotic effect.

15789

Growth of Chicks on Purified and Synthetic Diets Containing Amino Acids.*

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Although amino acids have been used in recent investigations[1-3] as the principal nitrogen source in chick diets in order to determine the amino acid requirements of the chick, such diets have various other applications in nutritional investigations. We have fed amino acid diets in an attempt to determine (1) if the amino acids present in casein can be replaced by an equivalent amount of free amino acids; (2) if chicks will grow as well on a mixture of essential amino acids as when additional nonessential amino acids are added; (3) if chicks require strepogenin;[4,5] (4) if chicks will survive and develop normally on a purely synthetic diet (a chemically-defined diet consisting of C.P. chemicals); and (5) if a chemically-defined diet can be used effectively in the elucidation of unknown factors such as the monkey antianemia factor of Cooperman *et al.*[6]

Experimental. Day-old White Leghorn chicks weighing 40-42 g were divided into groups of 3 chicks each and maintained in electrically-heated cages with raised screen bottoms. In Series 1 and 2 (Table II) the chicks were fed a protein and amino acid-free diet for 3 to 4 days (to deplete them of any possible nitrogen stores carried in the yolk sac) prior to feeding the experimental diets. Since this preliminary depletion period appeared to inhibit later growth responses it was discontinued and the animals were placed directly on the experimental diets in subsequent experiments.

The composition of the diets used is given in Table I. Diet I is a typical experimental diet with 18% casein supplemented with arginine, glycine, and cystine and adequate amounts of the vitamins known to be involved in chick nutrition. Diet II is similar to Diet I but contains free amino acids equivalent to the amount present in casein with arginine, glycine, and cystine additions as in Diet I. Diet III contains the amino acids essential to the chick in amounts adequate to equal the nitrogen contents of Diets I and II. Diets IV, V and VI represent

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and Swift and Company.

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TABLE I.
Diet Composition in Percent.

Constituent	I	II	III	IV	V	VI	VII
Dextrin*	61.0	61.0	61.0	33.0	33.0	33.0	
Cerelose				33.0	33.0	33.0	
Celluflour†				3.0	3.0	3.0	
Glucose C.P.							71.0
Soybean oil	5.0	5.0	5.0	5.0	5.0	5.0	
Salts V‡	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Haliver oil§	.04	.04	.04	.04	.04	.04	
B-vitamins¶							
Calcium gluconate				0.4	0.4	0.4	0.5
Casein (SMA)	18.0				8.0	14.0	
d,l-Alanine		1.23		0.2	0.1		0.2
l(+)Arginine HCl	0.6	1.59	2.4	1.2	0.6	0.6	1.8
d,l-Aspartic acid		1.38		0.2	0.1		0.2
l(+)Cysteine HCl		0.09					
l(—)Cystine	0.3	0.39		0.4	0.2	0.4	0.3
l(+)Glutamic acid		5.00		1.5	0.75		1.5
Glycine	2.0	2.11	2.0	1.0	0.5	1.0	1.8
d,l-Histidine HCl · H_2O		0.68	1.2	0.6	0.3		0.2
d,l-Homocystine		0.05					
l(—)Hydroxyproline		0.05		0.1	0.05		
d,l-Isoleucine		2.85	4.0	2.0	1.0		2.0
l(—)Leucine		2.66	3.0	1.5	0.75		1.5
l(+)Lysine HCl		1.88	2.5	1.2	0.6		1.8
d,l-Methionine		0.77	2.0	1.0	0.5		1.5
d,l-Norleucine		0.05					
d,l-Phenylalanine		1.13	3.0	1.0	0.5		2.0
d,l-Proline		1.80		0.2	0.1		0.2
d,l-Serine		0.11		0.2	0.1		0.15
d,l-Threonine		1.72	4.0	1.4	0.7		2.0
l(—)Tryptophane		0.26	2.0	0.5	0.25		0.5
l(—)Tyrosine		1.40		0.5	0.25		1.0
d,l-Valine		2.94	4.0	2.0	1.0		2.0
Total Nitrogen**	3.38	3.38	3.36	1.87	2.20	2.59	2.36

* Corn starch moistened with water and autoclaved at 15 lb for 2 hr, dried, and ground.

† A commercial cellulose product.

‡ Diets IV, V, and VI contained 0.5% Na_3PO_4 and diet VII contained $NaHCO_3$ 1.0%, Na_2HPO_4 0.5%, $Al_2(SO_4)_3$ 0.1%, $Na_2B_4O_7 \cdot 10H_2O$ 0.1%, $VaCl_2$ 0.05%, $NiSO_4$ 0.03%, $Co(NO_3)_2$ 0.03%, and K_2SiO_4 0.1% in addition to salts V.

§ Haliver oil fortified with vitamin D_3 to supply .006 mg/100 g ration.

¶ Diets I, II, and III contained α-tocopherol 0.6 mg, 2-me-1,4-naphthoquinone 0.1, thiamine Cl 0.5, riboflavin 1.2, nicotinic acid 10.0, calcium pantothenate 4.0, pyridoxine HCl 0.8, choline Cl 200.0, inositol 100.0, biotin 0.04, and folic acid 0.5 mg per 100 g of ration.

Diets IV, V, and VI contained double the above amounts of α-tocopherol, thiamine Cl, nicotinic acid, riboflavin, calcium pantothenate, pyridoxine HCl and biotin and 2-me-1,4-naphthoquinone 0.1, ascorbic acid 100 mg, *p*-aminobenzoic acid 5.0, folic acid 0.05, and *i*-inositol 100 mg per 100 g of ration and 0.2 mg cholic acid per 100 g of ration. Diet IV contained 200 and diets V and VI 300 mg respectively of choline Cl per 100 g of ration.

Diet VII contained carotene 0.6, ascorbic acid 100, vitamin D_3 .005, α-tocopherol 33, 2-me-1,4-naphthoquinone 2.0, thiamine Cl 0.5, riboflavin 1.0, nicotinamide 10.0, Ca pantothenate 2.0, pyridoxine Cl 1.0, pyridoxal Cl 0.1, β-pyracin Cl 0.5, choline Cl, 200.0, *i*-inositol 100.0, biotin 0.05, folic acid 0.05, and *p*-aminobenzoic acid 10.0 mg per 100 g of ration. In addition Diet VII contained ethyl linolate 0.1, tripalmitin 1.0, ribose 0.03, and arabinose 0.5 g and cholic acid .2 mg per 100 g of ration.

** Non-utilized forms of amino acids are omitted in this sum.

modifications of amino acid mixtures with and without protein supplementation. Diet VII is a chemically-defined diet that contains additional growth factors and trace elements. All differences in total weights of the nitrogen sources were compensated for by modifying the level of carbohydrate.

Results. A direct comparison of individual groups within a given series is difficult, but a comparison of similar lots in the first 3 series demonstrates that casein diets (Groups 1, 7 and 12, Table II) produce better growth

TABLE II.
Amino Acid Diets in Chick Growth.

Series No.	Group No.	Diet No. and description	% nitrogen	Days on experiment	G gain per day
1	1	I—casein, arginine, glycine, and cystine	3.38	18	6.7 (3)*
1	2	II—22 amino acids	3.38	18	2.9 (3)
1	3	III—11 essential amino acids	3.36	14	1.0 (3)
2	4	IV—19 amino acids	1.87	10	1.5 (2)
2	5	V—19 amino acids + 8% casein	2.20	10	2.0 (3)
2	6	III—11 essential amino acids	3.36	10	0.6 (3)
2	7	VI—casein, arginine, glycine, and cystine	2.59	10	4.7 (3)
2	8	VI—casein,† arginine, cystine, and glycine	1.30	10	1.8 (3)
3	9	IV—19 amino acids	1.87	8	3.9 (2)
3	10	V—19 amino acids + 8% casein	2.20	8	3.0 (2)
3	11	III—11 essential amino acids	3.36	8	1.6 (2)
3	12	VI—casein, arginine, glycine, and cystine	2.59	8	4.1 (2)
3	13	VI—casein, arginine, glycine, and cystine	1.30	8	2.6 (2)
4	14	VII—chemical diet—no protein	0.00	16	—0.5 (3)
4	15	VII—18 amino acids	2.36	16	0.7 (3)
4	16	VII—16% amino acids + 4% liver powder‡	1.82§	16	1.5 (2)
4	17	VII—16% amino acids + 4% casein	2.45	16	3.0 (3)
4	18	VI—casein, arginine, glycine, and cystine	2.59	16	5.8 (3)
4	19	VI—casein, arginine, glycine, and cystine	1.30	16	1.0 (2)
4	20	VII—12% amino acids + 4% liver powder + 8% casein	2.67	16	2.5 (3)

* Figures in parentheses refer to number of survivors at the conclusion of the experiment.
† Casein, arginine, glycine, and cystine mixture reduced by one-half and replaced by dextrin.
‡ Whole liver powder.
§ Nitrogen content of the whole liver powder is not included in this sum.

responses in chicks than diets containing the 11 essential amino acids plus nonessential amino acids (Groups 2, 4 and 9) and that the latter diets are in turn superior to diets containing the 11 essential amino acids alone (Groups 3, 6 and 11). Growth differences between similar groups in the different series are not significant since different depletion technics and different experimental times were involved. Furthermore, although uniform chicks were used in any given series, dietary differences in the diets of the hens used as the source of eggs could affect the stores of the chicks which would alter their growth responses on similar diets in series run at different times.

Substitution of one-half of the weight of the amino acids in Diet IV with an equivalent weight of casein (8%) (Groups 5 and 10) produced no better growth responses than the amino acid diets (Groups 4 and 9) or a casein diet in which the nitrogen level was reduced 50% by feeding one-half of the casein and supplementary amino acids of Diet VI (Groups 8 and 13).

Chicks fed an amino acid-free synthetic diet (Group 14) lived for a remarkably long period and displayed no macroscopic deficiency symptoms other than loss of weight and lethargy. Chicks fed the complete synthetic Diet VII (Group 15) showed poor growth responses. Addition of 4% whole liver powder at the expense of the amino acids doubled the growth response (Group 16). The use of casein in place of whole liver powder gave even better growth responses in the chicks (Group 17) while the addition of both liver powder and casein (Group 20) appeared to produce no better growth response than 4% casein alone.

Discussion. The maximum growth responses on our amino acid diets (3.9 and 3 g per day) compare favorably with those of Hegsted (2.7 and 3.5 g per day).[2] We recognize that an absolute comparison cannot be made since different sources of animals and different experimental periods were involved.

The general failure of the amino acid diets to produce growth responses equal to diets containing casein supplemented with arginine, glycine and cystine can be explained only partially by the lack of a growth factor since whole liver substance apparently failed to supplement casein (source of strepogenin) on our synthetic diet. Our results do not, however, eliminate an extremely labile growth factor absent from the whole liver powder used. It is doubtful that the growth differences are due to missing trace elements since the whole liver powder would probably carry adequate amounts of any unidentified essential minerals.

Growth inhibition due to toxicity of some of the *d*-isomers is debatable. Replacement of part of the amino acids in the diet containing the 19 amino acids by casein failed to produce a significant growth response in chicks over a reduced casein diet (Groups 5, 8, 10 and 13) which would be expected if toxic *d*-isomers were involved. This occurred in spite of the fact that some of the amino acids were borderline in the reduced casein diet. Alternatively, the poor growth responses obtained on the diet containing the 11 amino acids (3.36% nitrogen) when compared to the diets containing the 19 and 22 amino acids (2.20 and 3.38% nitrogen respectively) could be explained by the higher percentage of the *d*-isomers in the diet containing the 11 amino acids, or by the possibility that some of the so-called nonessential amino acids are essential on such highly purified diets. It is possible that the problem is physiological rather than nutritional and involves amino acid imbalances other than toxicity of the *d*-isomers, disturbed osmotic pressure relationships, interference with buffer mechanisms in the intestine, poor retention in the tract, or impaired absorption. Inhibition of enzymes or beneficial bacteria in the intestinal tract are other possible effects.

The role of strepogenin in the nutrition of the chick remains obscure. The addition of casein or whole liver powder to our synthetic diet (Diet VII) produced a positive strepogenin-like response on chick growth (Groups 14, 16, 17, and 20) in contrast to the results with the purified diet (Diet IV) where replacement of one-half of the amino acids with casein failed to give increased growth in the chicks. The slightly higher nitrogen content of the supplemented synthetic diets could have been a factor involved. It is entirely possible that factors other than strepogenin were lacking and it is questionable if uniform growth responses to strepogenin would occur on such a deficient diet. There is also the possibility that the chick carries adequate stores of strepogenin and a true strepogenin response could not be expected on such short term experiments necessitated by the high cost of amino acids.

Summary. Chicks fed a completely synthetic (chemically-defined) diet in which amino acids are the sole source of nitrogen show a definite subnormal growth response.

Casein is superior to its component amino acids as a source of nitrogen for the chick.

Diets containing some of the nonessential amino acids are superior to diets containing the 11 amino acids now recognized as essential for the chick.

We gratefully acknowledge our indebtedness to Merck and Company, Inc., Rahway, N.J., for crystalline vitamins; Wilson Laboratories, Chicago, Ill., for gelatin and whole liver powder; Allied Mills, Peoria, Ill., for soybean oil; Lederle Laboratories, Inc., Pearl River, N.Y., for folic acid; Hoffmann-LaRoche, Nutley, N.J., for *l*-proline; E. I. duPont de Nemours and Company, Inc., New Brunswick, N.J., for crystalline vitamin D_3; and Dr. H. R. Snyder of the Department of Chemistry, University of Illinois, Urbana, Ill., for *dl*-serine.

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Absence of Lipotropic Activity of Methionine When Pure Amino Acids Were Substituted for Dietary Protein.

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That methionine shows lipotropic activity in preventing the development of fatty livers has been repeatedly demonstrated, but the variation in experimental procedures and in the results obtained has left the magnitude of this effect and the interrelationships of methionine with other lipotropic and anti-lipotropic agents in some question. Lucas and Best[1] have reviewed this subject and have noted that there is disagreement among various workers as to whether the lipotropic action of casein is due solely to its methionine content. It was thought pertinent in this connection to observe the effect of methionine when a mixture of essential amino acids (supplemented with glutamic acid) was substituted for natural protein in the experimental diet.

Experimental. The composition of the amino acid mixture used is shown in Table I. It was designed to contain the essential amino acids (with the exception of methionine) in the proportions in which they occur in casein, glutamic acid making up the nonessential fraction. When the *l*-form was not obtainable, twice the amount of the *dl*-form was used.

TABLE I.
Amino Acid Mixture
(% Composition)

l-Arginine HCl	4.4
dl-Phenylalanine	10.3
dl-Leucine	27.0
dl-Isoleucine	9.7
l-Histidine HCl	2.3
l-Lysine HCl	7.0
dl-Threonine	8.6
dl-Tryptophane	2.4
dl-Valine	10.0
l-Glutamic Acid HCl	18.3

Thirty young adult male rats of a Wistar strain were divided into 2 groups of 15 rats each. All animals were fed on a diet consisting of amino acid mixture, 8.5; cane sugar, 47.5; Crisco, 40; salt mixture No. 2 (U.S.P. XII), 4. Each animal received daily by stomach tube 20 µg thiamine chloride, 100 µg calcium pantothenate, 20 µg pyridoxine and 25 µg riboflavin, dissolved in 1 cc of water. Three drops of percomorph oil furnishing 3750 units of vitamin A and 540 units of vitamin D, and 3 mg of α-tocopherol were given weekly. The methionine-treated group received an additional supplement of 50 mg *dl*-methionine dissolved as the sodium salt in 1 cc of water and administered by stomach tube.[2]

The rats were maintained on experiment for 21 days. Food intake and body weight were recorded daily. At the end of the experimental period the animals were exsanguinated by decapitation under light ether anesthesia and the livers removed, weighed and analyzed for total fat by the method of Tucker and Eckstein,[3] modified slightly. The kidneys were saved for histological examination.

Results and Discussion. The basal diet corresponded in essential amino acid content to one containing about 7% casein and, with the mixture of *l*- and *dl*-acids, to a little less than this in available nitrogen, with no sulphur-containing amino acid present. When supplementary methionine was given, the 50 mg per day represented a level in the diet of 0.8% on the basis of the average food intake of the group, or the same amount of methionine as a diet containing 25% casein.

That the rats should not thrive on the

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* The authors are grateful to Dr. Harry S. Goldblatt for examining the kidney sections and to Mrs. A. B. Stavitsky for technical assistance.

[1] Lucas, C. C., and Best, C. H., *Vitamins and Hormones*, Vol. I, p. 1, 1943, New York.

[2] Machella, T. E., and Griffith, J. Q., Jr., in Griffith-Farris, *The Rat*, 1942, Philadelphia.

[3] Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

sulphur-free diet was to be expected. Weight loss was continuous throughout the experiment averaging 30% for the group. The animals did not, however, exhibit any acute symptoms during the 21-day period. Methionine exerted a beneficial effect on the general condition of the animals. The methionine-supplemented group ate 15% more than the control animals. Weight loss paralleled that of the controls for the first week, but during the remaining 2 weeks of the experiment body weight was almost constant. The average weight loss for the period was 21%. This figure does not compare favorably with the average 12% loss of rats on an unsupplemented 8% casein diet which was observed in this laboratory.† This mild effect of methionine on body weight and food intake is in contrast with the results of Glynn, Himsworth and Neuberger.[4] These authors using a high carbohydrate, very low fat diet, with amino acids as the source of nitrogen, found that with the addition of 8 mg of methionine a day to the sulphur-free ration the rats ate two-thirds more, while 80 mg a day more than doubled the food intake. The variation was in the amount of carbohydrate consumed, the amount of amino acids being kept constant at 1.3 g per day. The animals used weighed 80 g at the beginning of the experiment. With no methionine, weight change in 21 days was —2.5%, with 8 mg of methionine, +2.5%, and with 80 mg of methionine, +44%. These workers were studying pathological changes in the liver. That this might be uncomplicated by fatty infiltration, they included in the diet sufficient choline to prevent fatty livers. Our diet was essentially choline-free which may have necessitated a quite different apportionment of the methionine among its various functions.

No necrosis was observed in any of the livers at autopsy, nor did the kidneys show any pathological changes.

Methionine proved quite ineffective as a lipotropic agent in our experiment. The level of liver fat in the control group, 19.7% was of the order usually obtained with a similar diet containing casein. The average for the treated animals was slightly lower, 17.9%, but the difference is insignificant. It has been reported many times that a similar amount of methionine supplementing a ration containing 5% casein has definite lipotropic effect. As examples may be cited the early paper of Tucker and Eckstein[3] in which it was stated that 0.5% of methionine added to the diet reduced the liver fat from 20.4% to 11.1% and the recent work of Clark, Eilert and Dragstedt[5] who found the liver fat changed from 31.5% to 9.3% by the same amount of methionine. In both cases the total level of methionine in the diet was lower than in our experiment: about 0.65% as compared with 0.8%. However, because of the better appetite of the animals on the casein diet, the actual amount of methionine ingested was a little greater than that given to our animals. The rats of Tucker and Eckstein received 71 mg per day and those of Clark, Eilert and Dragstedt, 58 mg, while ours had 50 mg.

The results of the present study appear to support the view that other factors than methionine enter into the lipotropic effect of a high casein diet, or even of a methionine-supplemented low casein diet. Under the conditions of the experiment methionine, in what has been considered as an adequate amount, gave no appreciable protection against development of fatty livers. The amino acid mixture used did not reproduce the composition of casein except for the essential amino acids. Some of the nonessential acids may play a role or, in their absence, the requirement for some of the essential acids may be increased. It has been suggested by du Vigneaud, Chandler, Moyer and Keppel[6] that in low protein diets a deficiency of some other precursor of choline than methionine may be the limiting factor of lipotropic activity. On the other hand,

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† Unpublished data.

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[5] Clark, D. E., Eilert, M. L., and Dragstedt, L. R., *Am. J. Physiol.*, 1945, **144**, 620.

[6] du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, 1939, **131**, 57.

TABLE II.
Effect on Body Weight, Food Intake, and Liver Fat of Adding Methionine to a Diet Free of Sulfur-Containing Amino Acids.

			Wt of animals			Liver	
Group	Treatment	No. of rats	Initial, g	Change, %	Food intake, g/day	Wt, g	Total lipid, %
I	Basal diet	15	160 (149-172)	—29.8 ± 0.8	5.5 ± 0.2	5.30 ± 0.24	19.7 ± 1.7
II	Basal diet + Methionine	15	164 (146-182)	—21.0 ± 0.8	6.3 ± 0.2	5.50 ± 0.14	17.9 ± 1.4

the amount of methionine required to show a lipotropic effect may be considerably increased in a diet containing minimal amounts of choline. In the present investigation crystalline vitamin B components were used, while in those quoted above[3,5] the animals were fed 0.5 g of yeast daily. This amount of yeast contains more than 1.0 mg of choline[7] which is an appreciable percentage of the daily requirement. It is possible, also, that some other component of yeast works in conjunction with methionine.

Summary. Under the experimental conditions chosen, methionine at a level of 50 mg per day (0.8% of the diet) did not prevent the development of fatty livers in rats fed a diet in which a mixture of essential amino acids replaced protein.

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15791

Implantation of Riboflavin Pellets in Animals and Man.

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Nutritional studies of the Palestinian population have shown that riboflavin deficiency is very often observed in this country,[1] and that its incidence is far more frequent than that of other vitamin deficiencies.[2] The clinical manifestations of this deficiency, such as glossitis, cheilosis, corneal vasicularization, etc., were particularly common in pregnant women. Low riboflavin excretion in the urine (averaging 95 γ per liter instead of 360 γ per liter in normal pregnant women) was found in 190 (21%) out of 900 pregnant women studied.[3] Furthermore, observations have been reported as to the harmful effect of riboflavin deficiency on the course of pregnancy (prematurity), on the condition of the fetus *in utero* (higher incidence of antenatal death) and upon the postpartum period (agalactia and hypogalactia).[4]

Surprisingly enough, these clinical manifestations appeared not only in the poorer classes, in which riboflavin intake was inadequate, but also in well nourished subjects. The fairly frequent incidence of riboflavin deficiency in well nourished individuals is principally due to inadequate resorption of this vitamin. The frequent occurrence of various intestinal and hepatic diseases (amebic dysentery, gastric achlorhydria and hypochlorhydria) and various forms of chronic hepatitis is undoubtedly the most important etiologic factor in the production of deficiency symptoms in these subjects. Riboflavin therapy generally gives striking results in those cases in which the deficiency is induced by insufficient intake.[1,3] On the other hand, poor results are generally observed when riboflavin is administered to subjects suffering from riboflavin deficiency due to inadequate resorption. In such patients the oral administration of riboflavin seems to be ineffective. Furthermore, riboflavin administered parenterally or intravenously, is eliminated from the body very rapidly. Thus, in order to maintain a constant level in the tissues and to prevent the rapid elimination of riboflavin,[3,4] the daily dose must be divided into many subdoses.

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[3] Braun, K., Bromberg, Y. M., and Brzezinski, A., *J. Obst. and Gyn., Brit. Empire*, 1945, **52**, 1.

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The poor therapeutic results obtained with riboflavin in patients suffering from diseases of the digestive tract led us to attempt the implantation of riboflavin pellets so as to afford adequate and continuous resorption of this vitamin. This method of supply seems to us particularly advantageous in patients who require repeated daily injections of riboflavin for a relatively long period. In addition, the difficulties involved in handling this substance which is easily inactivated when exposed to light, and the unreliability and lack of cooperation of patients, induced us to search means of supplying riboflavin in controlled quantities.

Technic. Pellets implanted in rats. Pellets containing riboflavin* adsorbed on Fuller's earth were used at first but later discarded, because they were completely absorbed in the body within a fortnight. Much better results were obtained when a mixture of 20 mg cholesterol plus 20 mg riboflavin was used. Pellets prepared in this way lost 90% of their riboflavin content within 1-1½ months. In the present report the results obtained with this type of pellet will be described. It seems that better results can be obtained with pellets prepared by melting together cholesterol with riboflavin. Results with this method will be described elsewhere. Sterilization of the pellets was carried out by heating them twice for 4 hours at 80°C.

Pellets implanted in patients (humans). The pellets implanted in patients were made by mixing 50 mg cholesterol with 50 mg riboflavin. This mixture was made up into 4 pellets. They were sterilized by heating them 3 times for 4 hours at 80°C. The riboflavin was handled in darkness; implantation was carried out subcutaneously in the lateral aspect of the thigh. Four pellets containing 50 mg riboflavin in all were implanted into each patient.

Riboflavin determination in the urine and the recovered pellets was carried out by the microbiological method of Snell and Strong.[5,†]

Results. Resorption of riboflavin pellets in rats. A sterile pellet containing 20 mg of riboflavin with 20 mg of cholesterol was implanted in the medial muscles of the thigh of 5 female albino rats weighing 150 g each. The rats were kept on a stock diet consisting of sprouted wheat, oats and bran supplemented by milk and vegetables in season. Riboflavin excretion in the urine was determined at intervals of 2 days for 2 weeks preceding the implantation. After the implantation riboflavin excretion was determined every other day, and when the riboflavin level had returned to normal, at weekly intervals. When the rats were sacrificed, the "ghost" of the pellet was recovered and its riboflavin content determined. It contained on an average 20% of the original riboflavin content one month after implantation. The results are compiled in Table I. Table I demonstrates that increased riboflavin excretion persisted for one month after implantation. Later on, riboflavin excretion returned to the normal level, thus proving the discontinuance of any further resorption of riboflavin from the pellet.

Resorption of riboflavin pellets in human patients. At first one normal well nourished

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* We are indebted to "Assia" Chem. Lab., Tel-Aviv, for a generous supply of riboflavin.

[5] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

† We are indebted to Dr. Lea Bichowsky for these analyses.

TABLE I.

Riboflavin Excretion in the Urine of 5 Adult Female Albino Rats Before and After Intramuscular Implantation of Pellets Containing 20 mg Riboflavin (with 20 mg Cholesterol). The figures are for gamma of riboflavin per liter urine.

Days before implantation	Rat No. 1	Rat No. 2	Rat No. 3	Rat No. 4	Rat No. 5
14	115	90	95	100	90
12	120	150	100	110	100
10	110	170	110	90	95
8	90	140	90	120	110
6	100	100	75	80	115
4	115	70	90	90	110
2	95	130	110	100	95
Days after implantation					
2	500	500	530	800	600
4	360	300	960	600	700
6	290	290	510	500	860
8	240	250	450	420	510
10	500	300	550	640	400
12	600	900	300	410	350
14	400	800	420	640	280
18	600	480	450	800	700
21	650	320	400	700	840
28	600	200	200	690	600
35	125	260	140	400	510
42		225	110	120	90
49		280		110	
56		170		90	
63		80		100	

woman was implanted with riboflavin pellets (4 × 25 mg) containing 50 mg riboflavin in all. The riboflavin excretion in a 24-hour specimen of urine was determined (Table II) at 2-day intervals for a period of 2 weeks before the implantation and was found to vary between 650 γ-1000 γ. The patient was kept on a normal diet which would have allowed for variations in the daily riboflavin excretion, between 250 γ-1000 γ per liter of urine. A distinct increase in the amount of riboflavin (1200 γ-2800 γ) excreted in this woman's urine persisted for approximately 1½ months following implantation. This result led us to implant riboflavin in a patient suffering from characteristic glossitis, seborrheic facial lesions, and ocular manifestations such as corneal vascularization. Previous treatment of this patient with orally administered riboflavin in daily doses of 20-30 mg was without any effect. These poor therapeutic results were explained by the existence of complete gastric achlorhydria and chronic diarrhea due to amebiasis. Parenteral riboflavin treatment consisting of 5 mg injections thrice daily gave moderately good but temporary results since 2 days after discontinuance of the treatment signs of riboflavin deficiency reappeared. Implantation of pellets containing 50 mg of riboflavin gave good results in this patient with complete absence

TABLE II.

Riboflavin Excretion in the Urine of a Normal Female Patient (A) and One Pregnant Patient (B) Suffering from Riboflavin Deficiency Before and After Intramuscular Implantation of Pellets Containing 50 mg Riboflavin (with 50 mg Cholesterol). The figures are for gamma of riboflavin per liter urine.

Days before implantation	Patient A	Patient B
14	870	100
12	900	150
10	650	120
8	800	200
6	950	250
4	1000	150
2	780	170
Days after implantation		
2	1200	1300
4	1800	1500
6	2000	1500
8	2400	2500
10	2800	3000
12	2000	3500
14	2600	3000
18	2200	3500
21	2500	3200
28	2000	2000
35	2000	1500
42	1500	1000
49	950	850
56	690	500

of deficiency symptoms for 2 months. At the same time the daily urinary excretion of riboflavin increased from the preimplantation level of $100\ \gamma$-$250\ \gamma$ to a concentration of $1000\ \gamma$-$3500\ \gamma$ per liter (Table II).

Discussion. The fact that it is possible to maintain a high riboflavin level within the body of animals and man, and to eliminate deficiency symptoms for a period of 1-1½ months, is encouraging. Cases are frequently encountered where regular oral intake of the required riboflavin is ineffective because of inadequate resorption in patients with gastric, intestinal or hepatic diseases. In such patients the riboflavin is only utilized when injected[6] in repeated daily doses. Since the maintenance of such treatment is often difficult, if not impossible, we believe that the implantation of pellets has a definite practical value. Furthermore, we intend to apply this method to other vitamins.

Summary. 1. A method is described for the preparation of riboflavin pellets that can be implanted in animals and in humans.

2. The effect of these riboflavin pellets (containing cholesterol) in cases of retarded riboflavin resorption lasts from 1-1½ months.

3. The riboflavin pellets implanted in a patient suffering from riboflavin deficiency brought about complete relief of relatively long duration from deficiency symptoms.

4. The method may be of practical value for patients suffering from riboflavin deficiency due to inadequate resorption, and when repeated injections are difficult to carry out.

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[6] Bicknell, F., and Prescott, F., *The Vitamins in Medicine*, London, W. Heinemann, Ltd., 2nd edit., 1946.

15792

Action of Sodium Salicylate on Hyaluronidase.

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The purpose of this communication is to report certain experiments on the inhibition of hyaluronidase by sodium salicylate *in vivo* and *in vitro*.

Despite wide therapeutic usefulness little is known concerning the mechanism of action of salicylates. A number of investigators have shown that sodium salicylate at relatively high concentrations ($0.05\ M$-$0.5\ M$), causes a reversible denaturation of certain biologically active proteins.[1–4] Euler and coworkers[5] found that salicylates inhibit certain enzyme reactions in concentrations as low as $0.01\ M$. Some of these are partially reversible by diphosphopyridine nucleotide. Ivanovics[6] observed that the growth of certain organisms such as *Staphylococcus aureus* was inhibited by concentrations as low as $0.002\ M$ while others such as *Proteus morganii* required much larger amounts. He correlated this difference with the ability to synthesize pantothenic acid and found that added pantothenic acid or to a lesser extent β,β-dimethyl-α-hydroxy-γ-butyrolactone was able partially to reverse the salicylate inhibition of *S. aureus*. He concluded that sodium salicylate owes its antibacterial effects to 2 different properties, namely specific inhibition of the synthesis of β,β-dimethyl-α-hydroxy-γ-butyrolactone and nonspecific protein denaturation.

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[2] Holden, H. F., *Austral. J. Exp. Biol. Med.*, 1937, **15**, 43.

[3] Bawden, F. C., and Pirie, N. W., *Biochem. J.*, 1940, **34**, 1278.

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[5] Euler, H. v. and Ahlstrom, L., *Z. physiol. Chem.*, 1943, **279**, 175.

[6] Ivanovics, G., *Z. physiol. Chem.*, 1942, **276**, 33.

Guerra[7,8] has recently reported that the intravenous injection of sodium salicylate causes an inhibition of the "spreading effect" of testicular extracts (hyaluronidase) in albino rabbits, as well as in patients either suffering from or having recovered from rheumatic fever. He has concluded that hyaluronidase is inhibited by sodium salicylate and that this activity is responsible for the therapeutic efficacy of this drug in the rheumatic state. This observation seems to be of the utmost importance since one of the most definite facts about rheumatic fever is the efficacy of salicylates in relieving certain symptoms of this disease. The further facts that pathologically this disease may be characterized as a disease of connective tissue and that the enzyme hyaluronidase attacks hyaluronic acid, a component of connective tissue, suggest a possible ray of light in the study of this enigmatic disease.

Duran-Reynals[9] has pointed out that many factors such as nutrition, age, and sex hormones influence the activity of hyaluronidase *in vivo*. It thus seemed of importance to determine whether sodium salicylate was actually an inhibitor of this enzyme or whether the effects observed by Guerra were due indirectly to changes produced in the animal. Consequently, after verifying the results of Guerra *in vivo*, we have conducted a series of experiments on the effect of sodium salicylate on the activity of hyaluronidase *in vitro*.

Testicular hyaluronidase was prepared from bull testis by the method of Hahn,[10] the purification being carried through the first ammonium sulfate fractionation. Our preparations had an activity of 145 viscosity-reducing units per mg N. Hyaluronidase from *Cl. perfringens* (NIH SR-12) was obtained by growing the organism according to the method of Rogers,[11] in a semisynthetic medium containing hyaluronic acid. The bacterial filtrate was dialyzed at 4°C previous to use. Such preparations had an activity of 500 viscosity reducing units per mg N. Hyaluronic acid was prepared from umbilical cords by a modification of the method of Haas,[12] using a much shorter extraction procedure in the Waring blendor.

Spreading activity was determined by injection into the skin of the back of albino rabbits, 0.25 cc of enzyme preparation mixed with 0.25 cc of unfiltered india ink (diluted 1:1). After 24 hours the animal was sacrificed and skinned. The area of spread on the inner surface of the skin was traced on to cellophane and measured with a planimeter. This area was compared to a control area in which india ink mixed with 0.85% saline was injected. This method allows a somewhat more accurate estimation of spreading than has been possible heretofore. Viscosity-reducing activity was measured essentially according to Haas,[12] and was expressed as the reciprocal of the half-life time × 10^3. It was found that when viscosity was plotted against the square root of the time a straight line function was obtained thus making the graphic determination of the half-life time more precise. All assays were done at 37°C. The mucin clot prevention test was found inapplicable to the salicylate experiments since the mixture of sodium salicylate with the horse serum albumin resulted in precipitation. The hydrolysis of hyaluronic acid was followed by the determination of N-acetyl-glucosamine by a modification of the method of Morgan and Elson[13] utilizing the Colman Junior Spectrophotometer at a wave length of 540 mμ. Salicylates were determined by the method of Brodie.[14]

The first experiments conducted were a repetition of those of Guerra using hyaluronidase prepared from *Cl. perfringens* cultures. Sodium salicylate was given both by mouth and intravenously in doses suffi-

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[8] Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

[9] Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

[10] Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1945, **21**A, No. 1.

[11] Rogers, H. J., *Biochem. J.*, 1945, **39**, 435.

[12] Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

[13] Morgan, W. T. J., and Elson, L. A., *Biochem. J.*, 1934, **28**, 988.

[14] Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Therap.*, 1944, **80**, 114.

TABLE I.
Reduction of Viscosity of Hyaluronic Acid by Hyaluronidase in the Presence of Sodium Salicylate.

Enzyme incubated with varying concentrations of sodium salicylate for 15 minutes and activity determined by viscosity reducing method. Inhibition is calculated by comparison with control under identical conditions of pH and salt concentration. (pH = 5.8.)

Enzyme	Concentration of sodium salicylate				
	0.20 M	0.10 M	0.05 M	0.025 M	0.013 M
	% inhibition				
Testicular	100	90	50	42	17
Cl. perfringens	100	75	43	19	0

TABLE II.
Release of N-Acetyl-Glucosamine by Hyaluronidase in Presence of Sodium Salicylate.
Enzyme incubated with hyaluronic acid (12 cc) in 0.2 M acetate buffer at pH 5.0.

Time (hr)	Control mg	0.12 M		0.09 M		0.06 M		0.03 M	
		*mg	†%	mg	%	mg	%	mg	%
		Testicular Hyaluronidase.							
1	300	55	82	120	60	185	38	235	22
2	330	55	84	130	60	230	30	240	27
4	400	55	86	175	54	260	35	320	20
		Cl. perfringens Hyaluronidase							
1	415	150	65	290	30	390	6	455	0
2	485	155	49	290	40	440	9	480	0
4	490	145	51	375	24	460	6	485	1

* Mg N-acetyl-glucosamine.
† % inhibition.

cient to attain a blood level of 20-30 mg per 100 cc of blood. Definite inhibition of spreading activity of hyaluronidase was obtained in the animals receiving salicylates. These results were similar to those reported by Guerra.

The next group of experiments was performed to determine the effect of sodium salicylate, *in vitro,* on both hyaluronidase prepared from *Cl. perfringens* filtrates and from bull testis. Graded amounts of salicylates were incubated with enzyme and at indicated times samples were withdrawn and assayed. Salicylates were added to the assay mixture in sufficient concentration to maintain the salicylate concentration constant. Total salt and hydrogen ion concentrations were kept constant.

Table I shows the composite results of a group of experiments in which activity was determined by the viscosity reduction method. It is evident that marked inhibition is obtained with a concentration of 0.05 M with both types of enzyme, but at lower concentrations only testicular enzyme was inhibited.

The inhibition of hyaluronidase by salicylates is reversible since removal of salicylate by dialysis produced almost complete restoration of activity.

The effect of salicylates on hyaluronidase was studied further by determining the effect of sodium salicylate on the liberation of N-acetyl-glucosamine. Enzyme was mixed with varying concentrations of sodium salicylate and hyaluronic acid and at definite intervals samples were withdrawn and N-acetyl-glucosamine was determined. Table II illustrates the results obtained under these conditions. It will be noted that a definite inhibition of release of N-acetyl-glucosamine from hyaluronic acid by both testicular and perfringens hyaluronidase is obtained. The degree of inhibition is apparently independent of time. Similar results were obtained with the viscosity reduction method. The inhibition of testicular enzyme has consistently been more complete and occurs at lower concentrations. This, it will be noted, is in conformity with the results obtained when the viscosity reduction assay has been used.

This observation is of interest in view of the recent reports of Haas[16] showing that hyaluronidase from different sources shows different sensitivity to antiinvasin, the hyaluronidase inhibitor present in normal blood. He has attributed these differences to the presence of other substances which he has termed proinvasin I and antiinvasin II. It is possible that our results depend on differences in purity of the various enzymes or merely chemical differences in the hyaluronidases from different sources. That such chemical differences exist is indicated by the work of Hahn.[10,15]

It is apparent from these data that sodium salicylate does exert an inhibitory effect on the activity of partially purified testicular and perfringens hyaluronidase. The specificity of this effect may be open to doubt in that the concentrations necessary are in the range in which sodium salicylate produces denaturation of other biologically active proteins. This concentration (0.01 M-0.1 M) is considerably above that obtained therapeutically (0.001 M-0.005 M). Our work and that of Guerra indicates that sodium salicylate at therapeutic levels does however affect the spreading activity of hyaluronidase. The direct inhibition of hyaluronidase at these concentrations would seem unlikely on the basis of the data presented above but cannot be ruled out in view of the impurity of the preparations used and the variability of sensitivity of hyaluronidases from different sources. The complex mechanism proposed by Haas[12] as well as the demonstration of the role of salicylates in inhibiting antibody formation[16] raise other possibilities as to the locus of action of salicylates on the hyaluronidase system.

Summary. Sodium salicylate has been found to inhibit the spreading effect of hyaluronidase *in vivo*. Hyaluronidase derived from *Cl. perfringens* and bull testis was also inhibited by sodium salicylate *in vitro*. The concentrations necessary for *in vitro* inhibition are considerably higher than those obtained *in vivo*.

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15793

Recovery of Herpes Simplex Virus from Rabbit Brain Nine Months After Inoculation.*

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We reported the precipitating effect of anaphylactic shock on latent Herpetic encephalomyelitis.[1] Further progress has been made in substantiating our original observations.[2] Although at that time we did not demonstrate the existence of the Herpes simplex virus in the central nervous tissues of the rabbits showing the encephalitic symptoms following anaphylactic shock, we pointed out the symptomatological and pathological identity of the disease produced with that of Herpes encephalomyelitis. We further reported the finding of intranuclear inclusion bodies in the brain of the rabbit. Experiments pointing out the production of encephalitic symptomatology and central nervous system pathology by various noninfective mechanisms[3-6] made it of considera-

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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[2] Campbell, B., and Good, R. A., unpublished MS.

[3] Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.

[4] Ferraro, A., *Arch. Neurol. and Psychiat.*, 1944, **52**, 443.

Protocol—Rabbit G 370.

11/8 to 11/12/45	One young adult albino rabbit was sensitized to egg white by administration of the following dosages on alternate days: 1 cc I.V.; .5 cc I.V.; and 1 cc I.M.
12/27	The rabbit was inoculated in the quadriceps muscle with 1 cc of a 10^{-1} suspension of H. F.$_{21}$ virus.
1/6/46	Definite evidence of encephalomyelitis, elevated temperature, paralysis of the right hind leg and encephalitic manifestations.
1/18	Temperature normal, no evidence of encephalitis, no paralysis. Apparent recovery from a mild Herpetic encephalomyelitis.
1/29	After temperature had been normal for 11 days and there was no evidence of encephalitis or myelitis the animal exhibited moderate anaphylactic shock following intravenous administration of .15 cc egg white.
2/7	Slight elevation of rectal temperature.
2/7 to 2/27	Development of full blown encephalomyelitis. The symptoms and signs included elevated temperature to 105.5, paralysis of both hind legs, weakness of front legs, excessive salivation, opisthotony, nystagmus, and facial twitching.
2/27	Temperature returned to normal and except for a few sequelae, animal essentially normal.
2/27 to 4/15	Temperature remained normal.
4/15	Mild anaphylactic shock.
4/22	Temperature elevated, nystagmus, increased salivation, gnashing of teeth and disorientation.
4/22 to 5/1	Elevated temperature and evidence of mild but definite encephalitis.
5/1 to 7/10	Essentially normal except for slight disposture of the head.
7/10	Severe anaphylactic shock produced by the intravenous administration of .3 cc egg white I.V.
7/18	Temperature elevation noted, animal began to appear ill.
7/18 to 8/30	Gradual progressive encephalomyelitis with very high fever and severe symptomatology.
8/30	Dead.

ble importance to know whether or not an active virus agent is present in the nervous tissues of the animals suffering acute exacerbations of encephalitis. Hurst, Cooke, and Melvin[7] reported the isolation of canine distemper virus from the central nervous system of dogs 6 weeks after a systemic distemper infection at a time when the dogs showed symptoms of central nervous system involvement. Brodie[8] and Bodian and Cumberland[9] have reported the recovery of poliomyelitis virus from the central nervous system of monkeys 16 and 14 days respectively after disappearance of the paralysis. The persistent excretion of polio virus in the stools of convalescents, Horstman and Melnick,[10] has received considerable attention.

This protocol illustrates the capacity of anaphylactic shock to precipitate latent virus encephalitis since on all 3 occasions when the animal was shocked symptoms and signs of encephalitis followed after a latent period of one week.

Recovery and Identification of the Virus. Under aseptic precautions samples of central nervous tissue were removed from the lumbar spinal cord, medulla oblongata, cerebellum, cerebral cortex and thalamus of Rabbit 370 and placed in separate test tubes containing 50% buffered glycerine solution (pH 7.4). After 2 days' storage at ice box temperature each sample of tissue was ground in a sterile mortar and suspended in saline to form a 1:10 dilution of rabbit brain.†

Three mice were inoculated with .03 cc of the tissue suspension from each of the above described areas of the rabbit's central nervous system. All the mice died within 3 days except those inoculated with the medulla oblongata tissue. On the third intracranial passage in mice a 10^{-1} suspension of the virus killed all mice inoculated on the second day after inoculation. A determination of the M.L.D.$_{100}$ showed it be .03 cc of a 1/10,000 dilution of mouse brain in normal saline.‡

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[5] Kabat. E. A., Wolf, A., and Bezer, A. E., *Science*, 1946, **104**, 362.

[6] Morgan, I., *J. Exp. Med.*, 1946, **85**, 131.

[7] Hurst, E. W., Cooke, B. T., and Melvin, P., *Aust. J. Exp. Biol. and Med. Science*, 1943, **21**, 115.

[8] Brodie, PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1238.

[9] Bodian, D., and Cumberland, *Am. J. Hyg.*, 1947, in press.

[10] Horstman and Melnick, *J. A. M. A.*, 1944, **126**, 1061.

† These suspensions showed no evidence of bacterial growth on blood agar streak plates or Brewer's medium deep cultures.

‡ This was the same M.L.D.$_{100}$ as the H.F.$_{21}$ virus possessed which was inoculated into G 370 nine months previously.

The virus, after a subsequent passage in mice, was inoculated onto the cornea of a young adult rabbit. This rabbit developed a kerrato-conjunctivitis typical of Herpes infection.§ After a period of 8 days this rabbit developed a fulminating encephalitis and died. Pathological study of the brain revealed findings typical of Herpetic encephalitis.

In another rabbit, No. 548, following the precipitation of active encephalomyelitis from the latent state by anaphylactic shock 120 days after inoculation of Herpes simplex virus into the quadriceps femoris muscle group, a virus agent was again recovered from the central nervous tissues. The technics of recovery of the virus and its identification by biological characteristics were the same as were used in Rabbit No. 370. The virus after 3 mouse passages was identical in all respects to the virus inoculated 4 months previously.

Summary. 1. An infectious agent was recovered from the central nervous system of a rabbit which had been inoculated 9 months previously with Herpes simplex virus and which had suffered 3 acute relapses of the disease. The virus recovered exhibited characteristics identical with those of the original virus. 2. A second instance of virus recovery 4 months after inoculation is reported.

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§ Corneal scrapings were obtained from the infected eye three days after inoculation and showed typical intranuclear inclusion bodies in the epithelial cells.

15794

Artifacts in Gold Shadowed Electron Micrographs Due to Electrons of High Intensity.

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The electron microscope has been used widely for the examination of virus preparations, for the sizes of many viruses are below the range in which the light microscope can be used to good advantage.[1] However, because of the low contrast which is afforded by small viruses, difficulties in securing good electron micrographs have been encountered. Electron microscope studies on small viruses were aided immensely by the development of the metal shadow-casting technic by Williams and Wyckoff.[2,3] This technic has also proved of value in securing good definition of virus particles in the presence of a background of material of smaller particle size. By means of this technic Sigurgeirsson and Stanley[4] and Oster and Stanley[5] have secured good delineation of the particles of tobacco mosaic virus in the unpurified juice and contents of hair cells, respectively, of plants diseased with tobacco mosaic. Although there was no difficulty in these studies in showing that most of the rods present in fresh virus preparations possessed a length of 280 mμ, occasional pictures were obtained which showed segmentation of the virus rods similar to that described by Williams and Wyckoff.[6] Further study has shown that this segmentation into shorter pieces of variable lengths is due to an artifact. This artifact and its controlled formation are described in the present paper.

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[3] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

[4] Sigurgeirsson, T., and Stanley, W. M., *Phytopathology*, 1947, **37**, 26.

[5] Oster, G., and Stanley, W. M., *Brit. J. Exp. Path.*, 1946, **27**, 261.

[6] Williams, R. C., and Wyckoff, R. W. G., *Science*, 1945, **101**, 594.

Methods. Tobacco mosaic virus from infected Turkish tobacco plants, isolated and purified by differential centrifugation,[7] was prepared in a suitable dilution and placed on the copper screens covered with a thin film of collodion in the normal fashion of preparing specimens for use in the electron microscope. The screens were then subjected to the oblique deposition of a very thin evaporated metal film after the manner described by Williams and Wyckoff. The apparatus used in this laboratory consists of a glass chamber mounted on a polished brass plate in which there are 4 supports for the 1 × 3 glass slides which carry the mounts. The chamber is evacuated to less than 10^{-5} mm. of mercury. The gold *used* for the shadow casting was deposited from a hot tungsten filament 20 cm. above and at an angle of 15° to the glass slides. The amount of gold deposited was controlled by observing the color of the light transmitted through the slide and comparing it to a sheltered "standard" slide placed within the chamber. Since the slides were arranged with their long axis toward the gold source, a gradation of gold was obtainable on each slide so that it was possible to select the screen with the desired amount of deposited gold.

An RCA Console Model (type EMC-1) electron microscope yielding a magnification of 5800 diameters was used for the observation of the specimens. In order to obtain the initial electron micrographs the minimum intensity that would still allow accurate focusing of the image on the fluorescent screen was used. After a suitable field had been procured, a micrograph was taken with as little delay as possible. The exact field was maintained and the intensity was raised to the maximum. Following the exposure of the specimen to the high intensity electron beam for such time as was necessary to secure the desired effect, usually about 5 minutes, a micrograph was again taken. In this manner it became possible to compare images of identical virus particles following exposure to low and to high intensity electron beams.

Discussion of Results. Fig. 1 shows the virus particles as they appear in a micrograph taken at low intensity. The background is uniform and the particles appear well defined. Fig. 2 shows the same particles after they had been subjected to the high intensity beam for 3 minutes. The background has become very granular and the virus particles appear segmented. This segmentation appears to be similar to that described by Williams and Wyckoff.[6]

Fig. 3 and 4 show another set of micrographs treated in a similar manner except that Fig. 4 was taken after 10 minutes of intense irradiation. It can be seen that, although the background has become quite granular, the degree of segmentation appears to be considerably less than in Fig. 2. Continued irradiation did not produce the extreme segmentation noted in Fig. 1. This may have been due to a difference in the thickness of the gold deposited on the surface of the particles. It can also be seen from a comparison of Fig. 3 and 4 that there are many particles missing or obscured in Fig. 4. The letters correspond to the same particle or group in each figure. There appears to be some correlation between the direction of the deposition of the gold in relation to the orientation of the particle and the obscuring of the particle.

Fig. 5 shows an extreme effect which can result from prolonged exposure at the maximum intensity. Some of the particles appear segmented and others show only a single very dense circular area. It seems likely that on prolonged exposure to a high intensity electron beam the gold becomes fluid and forms globules on the surface of the virus particle and on the collodion membrane. Since the gold globules may coalesce to different sizes, the resulting appearance may then vary from the smallest ones, giving the effect of segmentation of the particle, to the larger globules which are shown in Fig. 5. The fine beading or segmentation shown in Fig. 2 could easily be mistaken as evidence for the existence of small segments of the tobacco

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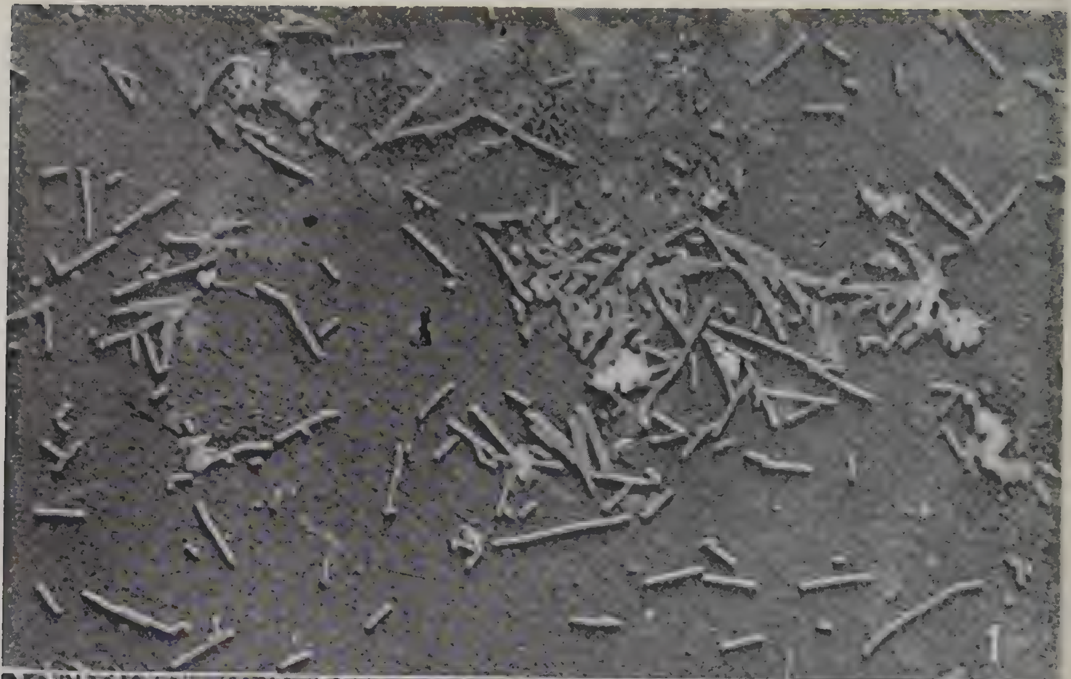
[7] Stanley, W. M., *J. Biol. Chem.*, 1940, **135**, 437.



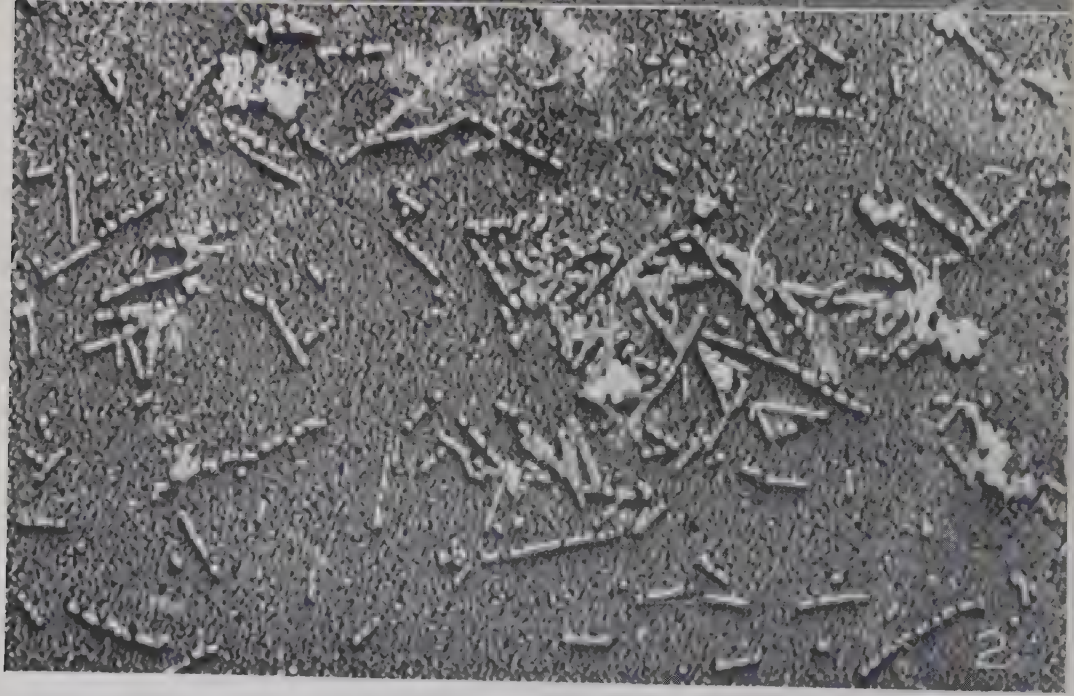
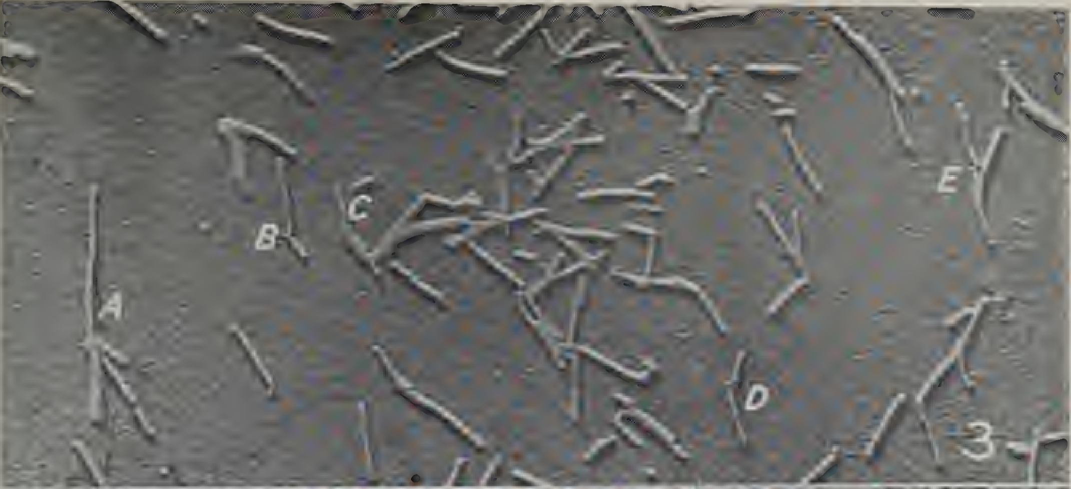
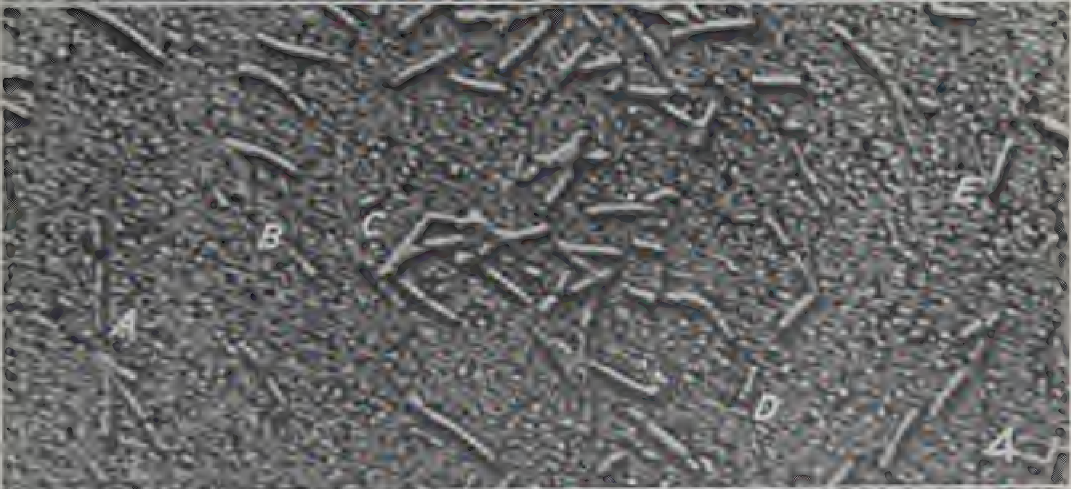


FIG. 1. Electron micrograph taken at low intensity of tobacco mosaic virus prepared with gold by the shadow-casting technic.

FIG. 2. The same specimen as that shown in Fig. 1 following exposure to a high intensity electron beam for 3 minutes. Many of the virus rods appear segmented.

mosaic virus rod. However it is obvious from Fig. 1 that the appearance of segmentation in Fig. 2 is an artifact resulting from exposure to the electron beam. It seems likely





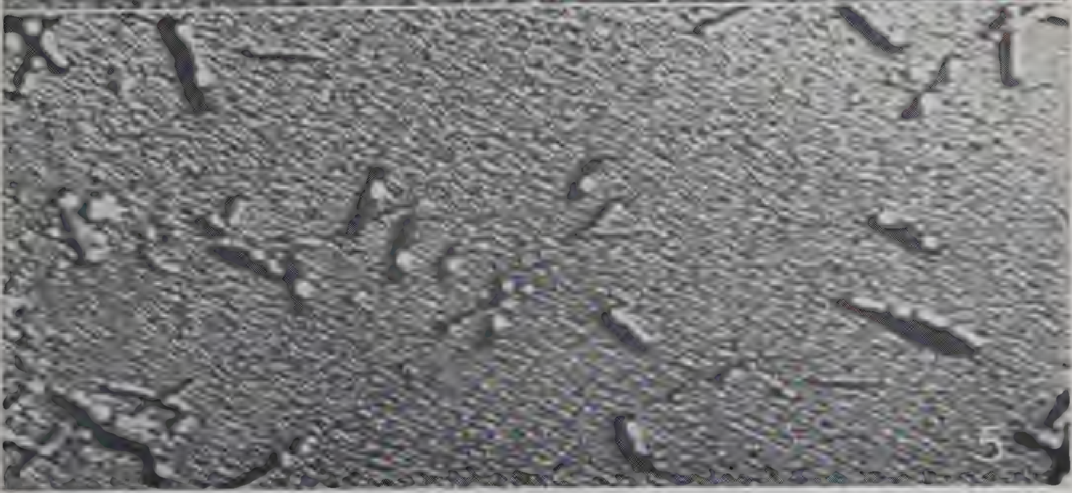


Fig. 3. Electron micrograph taken at low intensity of tobacco mosaic virus prepared with gold by the shadow-casting technic. Letters indicate identical rods in Fig. 4.

Fig. 4. The same specimen as that shown in Fig. 3 following exposure to a high intensity electron beam for 10 minutes. The exposure has caused the background to become granular and several of the particles are missing or not well defined.

Fig. 5. Tobacco mosaic virus prepared with gold by the shadow-casting technic after prolonged exposure to an electron beam at high intensity. The picture shows an extreme case of segmentation or bead formation.

that the segmentation of tobacco mosaic virus described by Williams and Wyckoff[6] was caused in a similar manner.

An effort was made to ascertain whether or not segmentation could be produced in tobacco mosaic virus preparations before shadow casting with gold or in preparations stained with phosphotungstic acid. No evidence for segmentation following prolonged exposure to a high intensity electron beam was obtained in either case. The results confirm the statement by Stanley and Anderson[8] for unshadowed material that "The fact that a micrograph taken with the first flow of electrons through a specimen does not appear to differ from subsequent micrographs taken after longer exposure to the electron beam makes it seem unlikely that gross changes are caused by the electrons."

The results of the present study indicate that in order to reduce or eliminate granulation and other obvious artifacts, which can occur when the shadow-casting technic with gold is used, it is necessary to avoid prolonged exposure of the specimen to the intense electron beam.

Summary. Electron micrographs of gold shadow-cast specimens of tobacco mosaic virus show that artifacts, consisting of segmented or even globular particles, are formed when the specimen is exposed to the beam of electrons at high intensity. It is suggested that these are caused by the thin film of gold becoming fluid and forming globules on the surface of the virus rods and supporting membrane. Tobacco mosaic virus not treated with gold did not show any change after having been subjected to the same treatment. The results indicate that gold covered specimens should not be subjected to prolonged exposure to an electron beam of high intensity.

It is a pleasure to acknowledge my indebtedness to Dr. W. M. Stanley for his active interest and helpful guidance.

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[8] Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

15795

Hypersensitivity to Egg White in the Rat.*

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Injection of egg white into previously sensitized rats elicits symptoms which have been taken as proof of an anaphylactic reaction.[1,2] Among these symptoms are hyperemia, cyanosis and edema. One author[1] mentioned that egg white has no primary toxicity. On the other hand it has been reported[3] that egg white administered parenterally is toxic and that sensitized and unsensitized rats reacted with almost identical symptoms. Among the symptoms there is a marked edema of the face, tongue, clitoris and paws preceded by hyperemia.[4] It seems therefore that the rat is naturally hypersensitive to egg white and that the symptoms attributed to anaphylaxis may be the result of this hypersensitivity. Since hypersensitivity is very seldom observed in animals from the first

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* These investigations were aided by a grant made by The Sugar Research Foundation to Dr. H. Selye.

† Fellow, National Research Council of Canada, Division of Medical Sciences.

‡ Fellow, Canada-Brazil Trust Fund, Instituto Butantan, Sao Paulo, Brazil.

[1] Molomut, N., *J. Immunology*, 1939, **37**, 113.

[2] Pratt, H. N., *J. Immunology*, 1935, **29**, 301.

[3] Parker, J. T., and Parker, F., Jr., *J. Med. Res.*, 1924, **44**, 263.

[4] Selye, H., *Endocrinology*, 1937, **21**, 169.

contact, this reaction offered an opportunity to approach it experimentally. The purpose of the present investigation is mainly to standardize the conditions under which this reaction will be further studied.

Experimental. In a preliminary experiment 12 male, hooded rats weighing 150-200 g were injected intraperitoneally with a dose of 3 cc of fresh egg white. The animals were examined 30 minutes, 2, 4 and 23 hours after the injection in order to determine when the reaction reaches its maximum. The only symptoms observed consisted of edema of the face, tongue and paws which was preceded by hyperemia noticeable mainly on the nose, ears and paws. The intensity of the edema, read separately for the face and paws, was recorded according to a scale from 0 to 3:0, no reaction; 1, slight but definite reaction; 2, moderate to marked reaction and 3, very marked reaction. The results were as follows: after 30′, incidence 15%, severity 12%; after 2 hours, incidence 100%, severity 84%; after 4 hours, incidence 100%, severity 70%; after 23 hours no more edema was visible. We therefore decided, in any further experiments, to examine the animals 2 hours after an intraperitoneal injection of egg white.

When the egg white is injected intravenously the symptoms appear more rapidly. Within a few minutes following injection there is hyperemia, followed by edema, which is maximum after 10 to 30 minutes. In some animals we noticed a cyanosis of the extremities, especially of the paws; in these cases there was no subsequent edema and the animals seemed otherwise normal.

It must be pointed out that this reaction does not apply to all proteins, because the injection of large doses of horse serum was without effect.

Having established that the rat is hypersensitive to egg white we devised another experiment in order to see whether any relationship existed between the dose of egg white injected and the incidence and intensity of the symptoms. Male albino rats weighing 60-80 g were divided into 6 groups of at least 10 animals each. Each group received intraperitoneally one injection of 1 cc, 0.5 cc, 0.25 cc, 0.15 cc and 0.10 cc of fresh egg white respectively. The animals were examined after 2 hours; the results are summarized in Table I.

TABLE I.
Effect of Dose of Egg White on Incidence and Intensity of the Edema.

Doses in cc	No. of animals	% incidence	% intensity
1.00	9	89	61
0.50	10	100	62
0.25	10	90	45
0.20	10	70	40
0.15	40	57	33
0.10	10	25	9

There is, evidently, a direct relationship between the dose and the response, which is more definite in regard to the incidence than to the intensity. The dose which gives approximately 50% of response is 0.15 cc.

In order to determine the variability of the response in the same animal and to see whether there is acquired tolerance, the animals of the latter experiment were injected, during 9 consecutive days, with the same initial dose of egg white. The responses were read 2 hours later. Since all the animals behaved similarly, we list in Table II only the observations on the first 4 animals of the groups receiving 1 cc, 0.5 cc and 0.10 cc of egg white.

From the results summarized in Table II it can be seen that: (1) the same intensity of edema is not necessarily present at the same time on the face and the paws or vice-versa; one animal may have a reaction of 3 on the face and no edema on the paws. There is no predilection for one area rather than for another. (2) The sensitivity of the animals varies from day to day. (3) On continuous treatment the sensitivity does not necessarily disappear.

Among the other variables, the composition of egg white should be taken into consideration. To eliminate this factor, we lyophylized a large quantity of egg white using it as a uniform stock for all these experiments.

It is known that age and sex have an influence on certain biological reactions. To in-

TABLE II.

Influence of Dose and Duration of Treatment on Production of Edema.

Group	Daily dose, cc	Rat	Day 1st	2nd	3rd	5th	6th	7th	8th	9th
I	1	A	3;3	0;0	2;2	2;0	0;1	0;0	0;0	0;0
		B	3;3	0;0	1;2	0;0	0;1	0;0	0;0	0;0
		C	0;0	1;0	0;0	0;0	0;0	0;0	0;0	0;0
		D	2;2	1;0	0;0	0;3	0;1	1;2	—	0;1
II	0.5	A	2;2	3;3	0;0	0;3	0;2	0;1	1;0	1;2
		B	1;2	0:1	0;0	1;3	0;0	1;0	0;0	0;0
		C	2;3	1:1	0;0	2;2	0;0	0;1	0;0	0;0
		D	3;3	1:1	0;0	3;2	1;0	0;0	0;0	0;0
III	0.25	A	3;2	0;1	0;0	1;0	0;2	0;1	0;0	0;1
		B	1;1	2;2	0;2	0;0	0;1	1;3	0;0	2;2
		C	3;3	0;0	1;0	2;0	0;0	0;0	0;0	1;2
		D	3;3	0;0	0;0	3;3	0;0	0;0	0;2	2;2
IV	0.10	A	0;0	0;0	0;0	0;0	0;0	0;0	1;1	0;0
		B	1;0	0;0	0;0	0;0	0;0	0;0	0;0	0;0
		C	0;0	0;0	0;0	2;0	0;0	1;3	0;0	0;0
		D	1;0	1;1	0;0	0;0	0;0	0;0	0;0	1;2

The first number in each column represents the intensity of edema on the face, the second on the paws.

TABLE III.

Influence of Age and Sex on Production of Edema.

			1st day		4th day		6th day	
			% incidence	% intensity	% incidence	% intensity	% incidence	% intensity
Group I	♂	40-50 g	89	37	25	12	75	33
Group II	♂	70-80 g	62	25	17	3	20	3
Group III	♂	120-140 g	75	37	12	4	29	7
Group IV	♀	120-140 g	37	19	12	4	25	11

vestigate this possibility we performed the following experiment. White rats were divided into 4 groups of 8 animals: Group I, male rats weighing 40-50 g; Group II, male rats weighing 70-80 g; Group III, male rats weighing 120-140 g, and Group IV, female rats weighing 120-140 g. All the animals received one intraperitoneal injection of 0.15 cc of egg white on the 1st, 4th and 6th day. The results are summarized in Table III.

Age evidently does not influence markedly the sensitivity of the animals; as far as sex differences are concerned it seems that the females are somewhat less sensitive although experiments with larger groups of animals would be necessary to provide definite proof of this point.

During the course of the present investigation we noticed that a few animals presented disturbances in the vestibular equilibrial apparatus resembling those seen in the Ménière's syndrome. Since they appear only after injection of egg white, the possibility of spontaneous otitis media can be eliminated.

Discussion. Judged by the present experiments, the egg-white reaction shows the following characteristics: (1) it occurs naturally in rats, (2) it is elicited at the first parenteral injection, (3) there is a direct relationship between dose and incidence or severity of the reaction, (4) the symptoms are always mild and restricted to certain areas: no case of severe reaction or fatal shock has been observed, (5) various areas are not necessarily affected to the same degree, (6) on continuous treatment the sensitivity of the animals varies from day to day; there is no permanent immunity.

It now remains to be determined whether these symptoms should merely be considered as part of a simple toxic reaction or classified among the phenomena of hypersensitiveness.

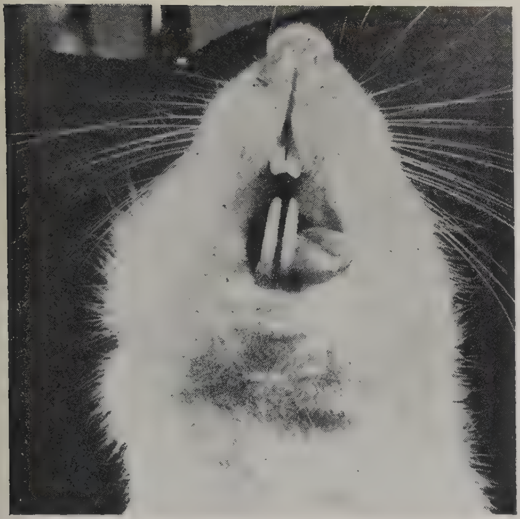

FIG. 1.
Normal control rat.

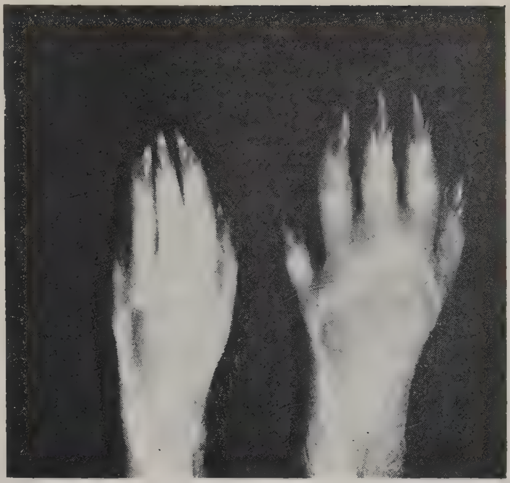

FIG. 3.
Left, paw of normal control rat. Right, edema of the paw after injection of egg white.

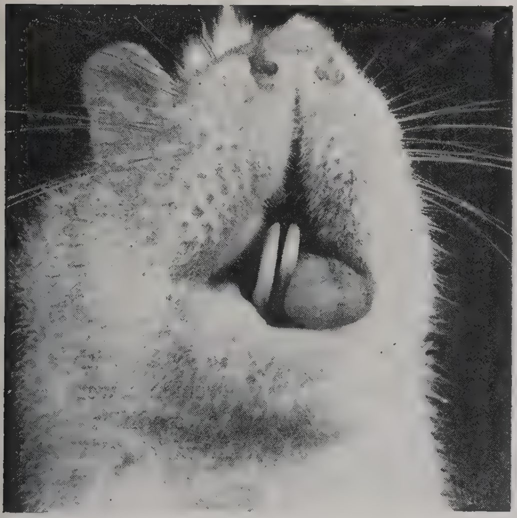

FIG. 2.
Egg-white treated rat. Note edema of tongue, nose and face. The two upper incisors are not visible because of edema and also the medial line of tongue is not as defined as in the control.

One author has already attributed the symptoms following parenteral injection of egg white to the toxicity of the substance.[3] Such a possibility is suggested by the relationship existing between dose and response. Against such an interpretation are the facts that even with high doses of egg white the symptoms are always mild and that the incidence and the severity of the reaction vary from day to day.

The reaction has some similarity with the local anaphylaxis and the symptoms elicited by the local injection or release of histamine. Against the anaphylactic theory is the fact that there is no need for a previous sensitization of the animals, and therefore no explanation can be based on an antigen-antibody mechanism. It seems to the authors that, tentatively, the egg-white reaction can be classified as an anaphylactoid phenomenon which is defined as a group of reactions resembling in their symptomatology those of true anaphylaxis but which cannot be referred to a specific antigen-antibody mechanism.[5] The inhibition of the egg-white reaction by antihistaminic substances gives some support to this interpretation.[6]

Although anaphylaxis is very difficult to elicit in the rat, the reaction to egg white can readily be obtained in a high percentage of the animals. It can be assumed therefore that different mechanisms are involved.

The symptoms obtained with egg white

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[5] Zinsser, H., Enders, J. F., and Fothergill, L. D., *Immunity. Principles and Application in Medicine and Public Health,* The Macmillan Co., New York, 1941.

[6] Leger, J., and Masson, G., in press.

are similar to those described for angioneurotic edema in man: edema of the face, lips, buccal cavity associated with vestibular crisis or other neurologic manifestations. Another similarity is the abrupt appearance of the symptoms and their sudden disappearance.

Summary. In the rat a single intravenous or intraperitoneal injection of egg white elicits a reaction characterized by hyperemia, followed by edema of the face, tongue and paws. Although only these regions respond, they are not necessarily affected to an equal extent. Continued injections of the same dose produce variable reactions in the same animals but there is no acquired tolerance. Age does not play a great influence, but females seem less sensitive than males.

The authors wish to express their sincere appreciation to Dr. M. Rocha e Silva for suggesting the subject of this investigation and for his helpful advice.

15796 P

Cholinesterase Content of Erythrocytes in Normal and Pathological States. I. Content in Normal Humans.*

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(Introduced by E. B. Carmichael.)

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Several investigators have recently reported changes in plasma, or whole blood cholinesterase content in anemias and leukemia. Values reported were expressed in terms of cholinesterase activity per volume of sample. Sabine[1] has calculated the cholinesterase content per cc of red cells but was unable to secure accurate values by direct measurement.

The red cells contain only specific cholinesterase and none of the nonspecific type found in plasma or serum. It is unlikely, therefore, that the cholinesterase in the red cells is derived from the circulating plasma, but, more probably, their esterase content is produced by the tissues from which they are derived or with which they are closely associated. Any change of esterase content per cell may be important, therefore, in reflecting the activity of the reticulo-endothelial elements of bone-marrow. The production of the esterase by the red cell itself cannot at this time be excluded.

Preliminary to some observations of the red cell content in various pathological states, we have determined the cholinesterase content per million cells in 18 normal human subjects. We have been able to secure accurate values by using volumes of washed, resuspended red cells calculated to contain the same number of cells as determined by cell count. The cells were lysed with 10% NaCl at 2°C. The measurements were then conducted at 37.5° by a modification of the method of Ammon[2] in which the bicarbonate-Ringers solution and the acetylcholine chloride solution were both placed in the large space and the blood sample in the side cup of the Warburg vessels. Values of cholinesterase activity thus secured are expressed as cmm of CO_2 produced per hour per million red cells. Esterase activity so expressed was not proportional to cell count or total blood hemoglobin. Values for different subjects varied from 3.94×10^{-4} cmm to 7.45×10^{-4} cmm with a mean value for normals of 6.16×10^{-4} cmm.

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* Aided by a grant from the Williams-Waterman Fund of Research Corporation.

[1] Sabine, J. C., *J. Clin. Invest.*, 1940, **19**, 833.

[2] Ammon, R., *Arch. ges. Physiol.*, 1934, **233**, 486.

15797

A Simple Effective Regimen for Preservation of the Dog With a Pancreatic Fistula.

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Dogs tolerate pancreatic fistulas poorly.[1] In addition to the chronic excoriation of the skin of the abdomen and legs, general physical deterioration is rapid. The anorexia, dehydration, hypochloremia, cachexia and ultimate death can be obviated by refeeding pancreatic juice or by parenteral administration of saline in addition to their regular diet.[1-3] The technical difficulties attending the maintenance of dogs with pancreatic fistulas is appreciated by anyone who has worked with these animals.

The author was confronted with a need for dogs with simple Pavlov type pancreatic fistulas which would survive for a prolonged period of time at a level of health compatible with the tolerance of major surgery (gastrectomy).[4] A simple effective maintenance regimen for these animals was developed, which successfully prevented skin excoriation and preserved the general health and vigor of these animals.

The skin of the abdomen and legs was protected from the irritative effects of pancreatic juice by maintaining the animals in trays of clean, fine, white sand. The dogs soon learned to keep their bellies nestled in this absorptive material, thereby obviating skin excoriation. It was necessary to change the sand twice daily.

TABLE I.
Daily Ration for Dog (Weight 15 kg) with Pancreatic Fistula.

Constituents	Quantity	
Raw pork pancreas	150	g
Beef tallow	40	”
Casein	100	”
Sucrose	100	”
Milk	500	”
Cod liver oil	4	cc
Brewers yeast	6	tablets
Vit. C	25.0	mg
Vit. K	1.0	”
Cowgills (2) salt mixture	4.14	g
Sodium bicarbonate	1.5	”
Bone ash	7.5	”
Amphogel	30.0	cc

Diet homogenized and administered by gastric intubation if necessary.

The daily replacement of the water, electrolytes, and sufficient pancreatic enzymes was met by the forced feeding of a ration of a homogenized liquid diet, nutritionally complete, for a dog with a pancreatic fistula. The dietary formula is listed in Table I. When necessary, in anorexic dogs, tube feeding insured complete ingestion of this diet. Antacids were added to prevent development of peptic ulcer incident to the loss of the normal neutralizing effects of pancreatic juice.[2]

Maintained on the above regimen, 5 of 6 dogs with pancreatic fistulas survived gastrectomy. The mortality in the 6th dog was due to a surgical blunder. Following gastrectomy, survival varied from 3 months to 2½ years. Deprivation of the special regimen with return to ordinary kennel care and rations evoked a dramatic deterioration and ultimate death in all of these gastrectomized dogs.

Summary. (1) A simple kennel and dietary routine for the maintenance of the health and vigor of dogs with pancreatic fistulas is outlined. (2) Pancreatic fistula dogs supported by this regimen tolerated major abdominal surgery (gastrectomy).

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* Fellow of the National Cancer Institute, Bethesda, Md.

The research presented here was supported by grants of the Graduate School of the University of Minnesota, the John and Mary R. Markle Foundation, the Augustus L. Searle Fund for Surgical Research, and the Citizens' Aid Society.

[1] Elman, R., and McCaughan, J. M., *J. Exp. Med.*, 1927, **45**, 561.

[2] Cowgill, G. R., *J. Biol. Chem.*, 1923, **56**, 725.

[3] Allen, J. S., Vermeulen, C., Owens, F. M., and Dragstedt, L. R., *Am. J. Physiol.*, 1943, **138**, 352.

[4] Kolouch, Fred, unpublished.

15798

Demonstration of Antibodies in Acquired Hemolytic Anemia With Anti-Human Globulin Serum.

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(Introduced by A. L. Bloomfield.)

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The technic described by Coombs, Mourant and Race[1] for detecting the presence of the heat stable or "blocking" Rh antibody by agglutinating the sensitized cells with an antiglobulin rabbit serum should be applicable to the investigation of other types of hemolytic disease in which the presence of an immune body is suspected. Agglutination of the sensitized cells depends on the interaction of the attached globulin of the blocking antibody and the antiglobulin rabbit antibody. It has been demonstrated by Boorman, Dodd and Loutit[2] that washed erythrocytes from patients with acquired hemolytic anemia are agglutinated in the antiglobulin rabbit serum whereas those from patients with familial hemolytic jaundice are not, indicating that only the former have a globulin antibody attached to the cell. Observations are reported here which indicate that the erythrocytes of 2 patients with acquired hemolytic anemia were coated with an immune body which could be released and transferred to normal cells *in vitro*.

Methods. The immune rabbit serum was produced by the intracutaneous and subcutaneous injection of 1 cc of group O human serum at weekly intervals for 2 courses of 4 injections each. The cell antibodies in the serum were absorbed by incubation with an equal volume of a 50% saline suspension of pooled A and B cells at 37°C for one hour. After 2 absorptions the serum did not agglutinate normal cells of any group or type, but when further diluted 1-10 with isotonic saline it produced agglutination of Rh_1 cells after they had been exposed to a 1-128 saline dilution of antiRh_0 serum containing only the heat stable or "blocking" antibody. The agglutinating titer of the antiRh_0 serum when diluted with 30% beef albumin was 1-128. Serial dilutions of the immune rabbit serum produced a visible precipitin reaction with a 1-10,000 dilution of commercial preparation of human immune globulin.

Erythrocytes were obtained by collecting capillary blood in normal saline or by defibrinating venous blood. The cells were washed 3 times with normal saline before suspending in the antiglobulin serum. The capillary tube technic[3] as well as the usual test tube method followed by centrifugation were used to detect agglutination and results were checked by observation of the suspensions on a glass slide.

The elution technic of Landsteiner and Miller[4] was used in the transference of the erythrocyte antibody as follows: Washed cells from the patient and from a normal group O individual were each suspended in twice their volume of normal saline and kept in a 56° water bath for 5 minutes with gentle agitation. The suspensions were then centrifuged quickly in heated cups and the supernatant fluid removed. The degree of hemolysis was observed to be roughly equal in the 2 samples. The eluate was divided into 1 cc amounts and 0.1 cc of a concentrated suspension of normal group O cells was added. The suspensions were kept over night at varying temperatures with intermittent agitation. At the end of this period samples were removed and the cells washed before suspending in the antiglobulin serum.

Results. Observations were begun with a patient who has been shown to hemolyze transfused cells as well as her own at an

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[1] Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.*, 1945, **26**, 255.

[2] Boorman, K. E., Dodd, B. E., and Loutit, J. F., *Lancet*, 1946, **1**, 812.

[3] Chown, B., *Am. J. Clin. Path.*, 1944, **14**, 114.

[4] Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, **42**, 853.

TABLE I.
Agglutination of Normal Erythrocytes by Anti-human Globulin Serum After Exposure to Eluate from Erythrocytes of a Patient with Acquired Hemolytic Anemia. Positive results were obtained only from suspensions which stood at ice box temperature. Agglutination of samples of the same suspension was increased by subsequent incubation at 37°C.

	Temp. incubation, °C	Agglutination in anti-globulin serum
Eluate from patient's cells	37	0
plus	20	0
0.1 volume normal O cells	5	+ (37°C 2 hr) ++
Eluate from normal cells	37	0
plus	20	0
0.1 volume normal O cells	5	0 (37°C 2 hr) 0

accelerated rate.[5] A hemolysin is presumed to be the cause of the hemolytic anemia which has continued without improvement. Her erythrocytes have shown a consistent agglutinability in all dilutions of the antiglobulin serum as high as 1-80 in contrast to the absence of agglutination in any dilution of normal erythrocytes or cells from patients with congenital hemolytic jaundice. It was noted in preparation of the eluate that exposure of the patient's cells to 56°C for 5 minutes removed or destroyed this agglutinable property.

When normal group O cells were suspended in the eluates prepared in the manner described above only those cells which had been suspended in the eluate from the patient's cells and kept at ice box temperature showed a tendency to agglutinate in the antiglobulin serum, while the cells suspended in the eluate from the control showed no tendency to agglutinate. Cell suspensions kept at room temperature and 37°C gave negative results. It was observed that agglutinability of normal O cells after exposure to the eluate from the patient's cells became more pronounced if, after removal from the ice box, a sample of the suspension was allowed to stand at 37°C for 1 or 2 hours before the cells were washed and suspended in the antiglobulin serum shown in Table I. These observations were repeated with freshly prepared eluate and cells of various types with the same results.

A second patient with an acute hemolytic anemia of the acquired type was studied and observations of similar nature have been made. She was 23 years of age and had no past or family history suggestive of hemolytic disease. For 3 weeks she had noted increasing weakness, nausea and yellow color. She was pale and jaundiced and the spleen was palpable below the costal margin. The hematocrit was 10, and Hb. 3.4 g/100 cc. Reticulocytes were 10% and the icterus index 50. Red cell fragility in hypotonic saline was greatly increased and spherocytosis was evident in the blood smear. She was group O, Rh_1. Repeated multiple transfusions had a temporary effect in elevating the erythrocyte count and hemoglobin and it seemed certain without more exact determinations that transfused cells were involved in the hemolytic process. Cold agglutinins were demonstrated in a titer of 1-32 and an atypical agglutinin active at 37°C was demonstrable for about 30% of O cells but had no Rh or MN specificity. An atypical hemolysin was not demonstrated.

This patient's erythrocytes showed marked agglutination in a 1-160 dilution of the antiglobulin rabbit serum even when observations were carried out at 37°C to eliminate the cold agglutinin as a factor. Her cells failed to show agglutination after exposure to 56°C for 5 minutes. Normal O cells from 3 individuals showed no agglutination in her serum, but when incubated in the eluate from her cells acquired a susceptibility to agglutination in the antiglobulin serum as shown in Table II.

Discussion. It is evident that the erythrocytes from the 2 patients with acquired hemolytic anemia had a property uniquely

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[5] Evans, R. S., *Arch. Int. Med.*, 1946, **77**, 544.

TABLE II.

Demonstration of an Immune Body in a Saline Eluate from the Cells of a Second Patient with Acquired Hemolytic Anemia. After 18 hr at 5°C the cells showed more intense agglutination in the anti-globulin serum after an additional incubation at 37°C.

	Group O cells 0.1 cc	Anti-globulin rabbit serum	
		18 hr ice box	+ 2 hr 37°C
Eluate from patient's cells 1.0 cc	1	±	++
	2	±	++
	3	±	++
Eluate from normal cells 1.0 cc	1	0	0
	2	0	0
	3	0	0

different from that of cells of normal individuals in being readily agglutinated by the antiglobulin rabbit serum. This susceptibility to agglutination has been observed so far only in cells that have been exposed to the Rh "blocking" antibody and presumably coated with an immune globulin which attaches to the cell but does not produce agglutination in saline suspension. This suggests that there is an immune body present in acquired hemolytic anemias which attaches to the cell but does not produce agglutination under conditions in which the cells are usually observed. This analogy was further borne out when it was observed that the washed erythrocytes from these patients became agglutinated when placed in 30% beef albumin and 2.0% acacia as are cells known to be sensitized with the Rh "blocking" antibody.[6,7] However, the patient's cells did not agglutinate when suspended in human serum as do cells sensitized by the Rh "blocking" antibody.[8]

The failure to remove the factor responsible for the agglutination of the cells by repeated washings and the ready removal by heating to 56°C again suggests a cell antibody combination.[4] In addition, the demonstration that a substance was released at 56°C which was then capable of attaching to normal cells and withstanding repeated washing further identifies the substance as an erythrocyte antibody. The transfer of the Rh blocking antibody has been carried out by this method.[9,10]

Only tentative explanation can be offered for the temperature effects which seemed to be important in the transfer of the antibodies to normal cells. It is possible that adsorption of the immune globulin on the cells proceeded better in the cold and that subsequent incubation at higher temperatures was necessary for fixation so as to prevent removal by repeated washing.

Although an antibody could be detected on the surface of the erythrocyte from the 2 patients it could not be demonstrated in the serum. The first patient has never shown evidence of an atypical agglutinin or hemolysin in the serum, and moreover group O cells which were suspended in her serum at 37°C failed to agglutinate when washed and suspended in the antiglobulin serum. The second patient exhibited a relatively low titer of cold agglutinins and an atypical agglutinin of an undetermined specificity active at 37°C. The origin of this atypical agglutinin is not clear since the patient had not had transfusions prior to the onset of her illness and a single pregnancy had terminated at the end of 4 months. While the specificity of this agglutinin remained to be determined there is evidence that it was not responsible for the hemolytic process. The group O cells which were not agglutinated by the patient's serum and judged to be compatible were washed and tested with the antiglobulin

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[6] Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

[7] Levine, P., *Am. J. Clin. Path.*, 1946, **16**, 597.

[8] Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

[9] Haberman, S., and Hill, J. M., *Texas State J. Med.*, 1944, **40**, 182.

[10] Carter, B. B., and Loughrey, J., *Am. J. Clin. Path.*, 1945, **15**, 575.

serum for the attachment of an incomplete antibody with negative results. Although these cells failed to show any evidence of sensitization after exposure to her serum *in vitro* at 37°C it was evident after repeated transfusions that these "compatible" cells shared in the rapid hemolysis *in vivo*. In other words, they were being destroyed by an immune body that could not be demonstrated in the patient's serum. It is probable that the immune body which caused the accelerated hemolysis in both patients was not demonstrable in the serum because it is adsorbed as it is produced by the large amount of cell antigen present.

It is not certain, however, that the particular body which was attached to the erythrocytes of both patients could not produce hemolysis or agglutination if present in sufficient concentration. Even if it is passive in this regard, the adherence of a protein to the cell surface probably exerts a detrimental effect by interfering with osmotic equilibrium. The spherocytosis observed may be produced by the accumulation of metabolites which raise the internal osmotic pressure and cause the absorption of fluid, thereby increasing osmotic and mechanical fragility of the erythrocyte and hastening its destruction. The failure to obtain agglutination of erythrocytes from patients with congenital hemolytic jaundice is compatible with the observations of Dacie and Mollison[11] that transfused cells have a normal longevity in this disease which suggests a cell defect rather than a hemolytic agent as the underlying cause.

Summary and Conclusions. The erythrocytes of 2 patients with acquired hemolytic anemia and spherocytosis have been shown to be readily agglutinated by an immune rabbit serum containing an antihuman globulin antibody. Since both patients have exhibited abnormal hemolytic activity for transfused cells this finding is compatible with the existence of an immune body type of hemolytic agent as the cause of the accelerated hemolysis.

Evidence has been presented to show that the immune substance can be separated from patients' cells by heating the cells to 56°C for 5 minutes and can then be combined with normal cells. The similarity of this immune body to the Rh blocking antibody is discussed. The demonstration of the presence or absence of an immune body attached to the erythrocytes should become a routine procedure in the study of hemolytic anemias.

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[11] Dacie, J. V., and Mollison, P. L., *Lancet*, 1943, **1**, 550.

15799

Role of Adrenalin in Recovery of Inhibited Conditioned Reactions.

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It was shown in earlier papers from this laboratory[1,2] that insulin coma and chemically- or electrically-induced convulsions cause the reappearance of previously inhibited conditioned reactions (c.r.). No distinct effect was exerted on positive c.r.'s by these procedures.[3,4] Since it had been shown previously[5,6] that the action which these proce-

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* Aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Gellhorn, E., and Minatoya, H. J., *J. Neurophysiol.*, 1943, **6**, 161.

[2] Kessler, M., and Gellhorn, E., *Am. J. Psychiat.*, 1943, **99**, 687.

[3] Gellhorn, E., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 155.

[4] Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1946, **56**, 216.

[5] Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1938, **40**, 125.

dures have in common is the excitation of the centers of the sympatheticoadrenal system, it was thought that the restoration of inhibited c.r.'s was likewise due to this increased reactivity of sympathetic centers. In order to clarify further the mechanism involved an attempt was made to determine whether the hypothalamic-cortical discharge, which occurs on excitation of diencephalic autonomic centers, (Murphy and Gellhorn)[7] or the release of adrenalin was responsible for the reestablishment of inhibited c.r.'s. For this purpose 2 series of experiments were performed. In the first, adrenalin was injected into normal animals whereas in the second group insulin coma was produced in adrenodemedullated animals. In both groups the effect of these procedures to restore c.r.'s which had been inhibited by lack of reinforcement was studied.

Methods. All experiments were performed on rats in which a definite escape reaction, as used in our earlier studies, had been established in response to the conditioned stimulus (bell or light). After the c.r. had been established it was inhibited by lack of reinforcement. Adrenalin (.2 mg per kg) was injected intraperitoneally 3 or 4 times on alternate days. The effect of these injections was compared with that induced by insulin coma or electroshock on 6 normal animals. The action of insulin coma was likewise studied in 6 adrenodemedullated rats. For further detail compare the literature cited above and the paper by Arnett and Gellhorn.[8]

Results and Discussion. Nineteen injections of adrenalin were performed on 6 normal rats in which preceding or following the adrenalin series the response of the rat to insulin coma or electroshock was studied. In all but one experiment no effect was elicited on the inhibited c.r.'s by the injection of adrenalin although the animals showed the typical recovery reaction under the influence of insulin coma or electroshock. In the one case in which the response of the c.r.'s was increased to 50% by adrenalin the effect was restricted to one day whereas a single injection of insulin leading to convulsion elicited in the same animal a marked recovery of the c.r.'s which lasted for 4 days and reached 90%. The experiments performed on adrenodemedullated rats led to a recovery (50% to 90%) of the c.r.'s after induction of an insulin coma. These experiments prove conclusively the fundamental difference between the effects of injected adrenalin and those of insulin coma and electroshock. Since in the latter conditions adrenalin is likewise involved, being secreted as the result of excitation of sympathetic centers, the difference, as far as restitution of inhibited c.r.'s is concerned, is based on a central action which occurs in insulin coma and electroshock and is not related to adrenalin.

The answer to this problem seems to lie in altered hypothalamic-cortical relations. Obrador,[9] Kennard,[10] and Murphy and Gellhorn[7] have demonstrated the profound effect which stimulation and destruction of the hypothalamus exert on electrical cortical activity. It may be assumed on this basis and on clinical evidence concerning autonomic relations in psychotic patients[6] that quantitative alterations in the activity of autonomic centers will in turn influence cortical functions.

It may be said that the reactivity of autonomic centers is greatly increased in insulin hypoglycemia[11] and in experimental convulsions.[12] This must lead to quantitative changes in cortical activity which may be the basis of alterations in behavior. Adrenalin, however, whether injected or secreted, far from causing a similar reaction, seems to depress autonomic centers and may be looked upon as a means of restoring increased excitability of these centers to normal (homeostasis, Darrow and Gellhorn).[13]

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[6] Gellhorn, E., *Autonomic Regulations*, New York, 1943.

[7] Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 341 and 431.

[8] Arnett, V., Kessler, M., and Gellhorn, E., *Am. J. Physiol.*, 1942, **137**, 653.

[9] Obrador, S., *J. Neurophysiol.*, 1942, **6**, 81.

[10] Kennard, M. A., *J. Neurophysiol.*, 1943, **6**, 405.

[11] Gellhorn, E., Ingraham, R. C., and Moldovsky, L., *J. Neurophysiol.*, 1938, **1**, 301.

[12] Gellhorn, E., and Darrow, C. W., *Arch. Internat. Pharmacodyn.*, 1939, **62**, 114.

The statement by Himwich and Fazekas[14] that my theory of the shock treatment in schizophrenia does not hold since adrenalin is ineffective in the treatment of schizophrenics[†] is obviously based on a gross misunderstanding. The core of the theory (cf. also Gellhorn[4,6]) is not that insulin coma and electrically- or chemically induced convulsions act through the secretion of adrenalin but that they greatly increase the reactivity of autonomic centers which in turn may influence the brain in such a manner as to restore normal behavior.[‡] The neurological basis for such a hypothalamic-cortical relationship has been elucidated by Murphy and Gellhorn whereas the changes in conditioned reflexes described in this series of papers indicate at least one of the mechanisms which contribute to an altered behavior.

Summary. Conditioned reactions which had been inhibited by lack of reinforcement in rats may be restored by insulin coma in normal as well as in adrenodemedullated rats. Apparently a secretion of adrenalin which is observed under conditions of insulin coma and electroshock does not play an integral part in the mechanism of the restoration of inhibited conditioned reactions. This interpretation is supported by the fact that the injection of adrenalin remains ineffective in the normal animals as far as restoration of inhibited conditioned reactions is concerned, whereas insulin coma and electroshock restore these reactions. It is inferred from these experiments that the restitution of inhibited conditioned reactions is linked up with a hypothalamic-cortical discharge previously established by Murphy and Gellhorn and is not due to the liberation of adrenalin which invariably accompanies the excitation of hypothalamic centers under conditions of insulin coma and electroshock.

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[13] Darrow, C. W., and Gellhorn, E., *Am. J. Physiol.*, 1939, **128**, 185.

[14] Himwich, H. E., and Fazekas, J. F., *Arch. Neurol. and Psychiat.*, 1942, **47**, 800.

[†] The paper by Dynes and Tod[15] to which they refer does not give any evidence for or against the adrenalin treatment of schizophrenics but points out that adrenalin injections cause less emotional reactions (anxiety and fear) in the schizophrenic than in the normal individual.

[15] Dynes, J. D., and Tod, H., *J. Neurol. and Psychiat.*, 1940, **3**, 1.

[‡] The secretion of adrenalin is but one symptom of this central effect and probably of minor importance.

15800

Biological Value of Proteins Determined With *Tetrahymena geleii* H.[*]

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The possibility that the ciliated protozoan, *Tetrahymena geleii* H, might be used to determine the biological value of proteins was suggested by the observations that this small animal utilizes unhydrolyzed protein[12] and that its amino acid requirements[13] approx-

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[*] Paper 40. For Paper 39, see Rockland and Dunn.[1] This work was aided by grants from the National Institute of Health and the University of California.

The biological value of proteins has been determined with chickens, dogs, rabbits, rats, swine, and humans by the classical McCollum, Osborne and Mitchell methods as well as by newer technics described by Cannon *et al.*[2] and by Harrison and Long.[3] Reviews have been given by Mitchell,[4] Madden and Whipple,[5] Barnes *et al.*,[6] Cahill,[7] Boas-Fixen,[8,9] Allison *et al.*,[10] and Block and Mitchell.[11]

[1] Rockland, L. B., and Dunn, M. S., in press (No. 39).

[2] Cannon, P. P., Humphreys, E. M., Wissler, R. W., and Frazier, L. E., *J. Clin. Invest.*, 1944,

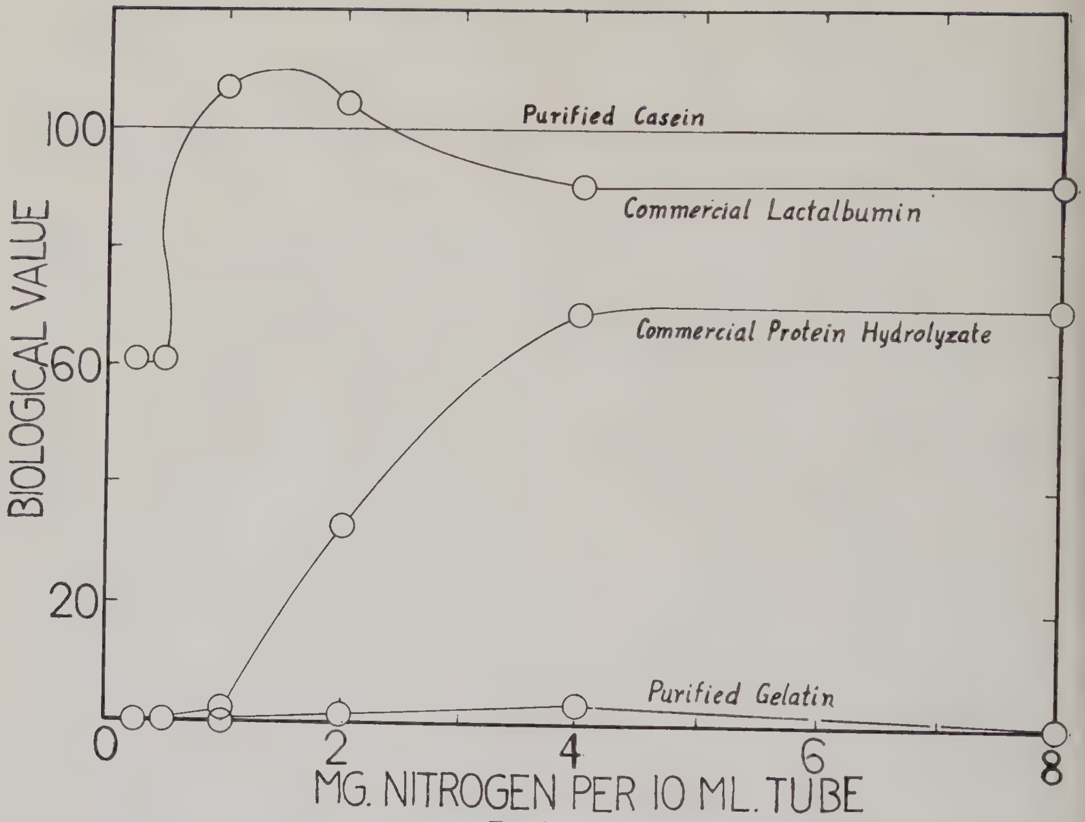

FIG. 1.
Curves relating biological value and nitrogen of proteins.

imate those of some mammals. It seems evident that biological values could be determined with greater speed, convenience and economy with this organism than with larger animals and that errors due to biological variation would be minimized because of the large number of individuals and the aseptic conditions employed.

Experimental. A protein-free basal medium similar to that described by Kidder and Dewey,[13] except for the omission of the amino acids, was employed in the present experiments. The technics were similar to those used in the authors' laboratory for the determination of amino acids with lactic acid bacteria. Aliquots of protein solutions, homogenized suspensions or hydrolyzates were added to 19 x 100 mm pyrex test tubes. Ten levels of sample, each in 2 or more tubes, were used. Protein- and amino acid-free medium and distilled water were added to a total volume of 10 ml per tube. The tubes were plugged, sterilized, inoculated with a

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23, 601.

[3] Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, 1945, **161**, 545.

[4] Mitchell, H. H., *Physiol. Rev.*, 1924, **4**, 424.

[5] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

[6] Barnes, R. H., Bates, M. J., and Maack, J. E., *J. Nutrit.*, 1946, **32**, 535.

[7] Cahill, W. M., *J. Am. Diet. Assn.*, 1945, **21**, 433.

[8] Boas-Fixen, M. A., *Biochem. J.*, 1934, **28**, 592.

[9] Boas-Fixen, M. A., *Nutr. Abstr. Rev.*, 1934-35, **4**, 447.

[10] Allison, J. B., and Collaborators, *Annals New York Acad. Sci.*, 1946, **47**, 241.

[11] Block, R. J., and Mitchell, H. H., *Nutr. Abstr. Rev.*, 1946-1947, **16**, 249.

[12] Rockland, L. B., and Dunn, M. S., *Arch. Biochem.*, 1946, **11**, 541.

[13] Kidder, G. W.; and Dewey, V. C., *Physiol. Zool.*, 1945, **15**, 136.

TABLE I.
Biological Values of Three Proteins.

Criterion	Animal	Protein Casein	Lactalbumin	Gelatin	Reference No.
Liver wt regeneration	Rat	100	100	0	3
Nitrogen balance	Dog	100	137		14
Plasma protein regeneration	,,	100	83		15
Protein level for optimum wt increase	Rat	100	133		16
Wt increase	Chick	100		46	17
,, ,,	Rat	100	100-206*		16
Acid production	*T. geleii* H	100	61-112*	0-2*	This paper

* Range of values at different levels of sample.

washed suspension of *Tetrahymena geleii* H cells, and incubated at about 25° (room temperature) for varying lengths of time. The response of the organism was measured by electrometric titration with standard base.

Discussion. The biological values were calculated from the titration data using casein as the standard. The values found for lactalbumin, a protein hydrolyzate and gelatin varied with the incubation time and, as shown in Fig. 1, with the level of nitrogen in the sample. It has been shown previously by McCollum *et al.*[18] and Plimmer *et al.*[19] that biological values vary with the time and by Osborne *et al.*,[16] Barnes *et al.*[6] and other workers cited by these authors that they vary with the level of the protein.

The present data for 3 proteins, as well as the values reported in the literature, are summarized in Table I. The biological values found at some levels of samples by the authors' micro method are in approximate agreement with those obtained by other procedures. Although this correlation is of interest, it appears more significant that the true biological value of a protein is best expressed by a series of numbers if time, level of sample and possibly other variables are to be taken into account. It may be suggested that biological values of most meaning may be represented by areas under curves or volumes under planes. Whether or not there is a direct, or any, relation between the human requirements for proteins and the biological values of proteins determined with protozoa or other animals remains to be determined.

Further studies of this problem are in progress.

Summary. A method for the determination of biological values of proteins with *Tetrahymena geleii* H has been described. The values found for casein, lactalbumin, a commercial protein hydrolyzate and gelatin have been compared with each other and with those reported in the literature.

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[14] Melnick, D., and Cowgill, G. R., *J. Nutrit.*, 1937, **13**, 401.

[15] Melnick, D., Cowgill, G. R., and Burack, E., *J. Exp. Med.*, 1936, **64**, 897.

[16] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, **37**, 223.

[17] Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R., *J. Nutrit.*, 1935, **10**, 193.

[18] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, **23**, 231.

[19] Plimmer, R. H. A., Rosedale, J. L., Raymond, W. H., and Lowndes, J., *Biochem. J.*, 1934, **28**, 1963.

15801 P

Treatment of Paroxysmal Auricular or Nodal Tachycardia With the Vasopressor Drug, Neosynephrine.

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The cardio-inhibitory reflexes elicited by a rise in blood pressure are strikingly sensitive in unanesthetized animals.[1] Therefore, it was considered that a moderate, sudden, brief rise in blood pressure produced by intravenous injection of a vasoconstrictor drug might reflexly stop a paroxysmal auricular or nodal tachycardia.

Neosynephrine (laevo-α-hydroxy-β-methylamino-3-hydroxy-ethylbenzine hydrochloride) has a relatively high pressor potency as compared with its direct cardiac stimulating potency.[2,3] Following intravenous injection in normal human subjects, an amount which produces a moderate increase in blood pressure causes a striking bradycardia.[4,5] Therefore, neosynephrine was chosen as a favorable vasopressor compound to be given a trial in the treatment of paroxysmal tachycardia. Neosynephrine has been given to several patients having cardiac arrhythmias, including 2 with paroxysmal auricular tachycardia, and these 2 reverted to normal sinus rhythm.[4]

Results. Neosynephrine was given intravenously to each of 4 patients with idiopathic paroxysmal tachycardia of supraventricular origin. Each had a normal blood pressure between attacks and a somewhat lower blood pressure during attacks. Electrocardiographic records were taken and blood pressure was recorded repeatedly during and following the injections with the patient in the supine position. In each case the neosynephrine injection was completed within 30 seconds. The first patient had tachycardia which had persisted for several hours in spite of attempts to stop it with carotid sinus pressure, ocular pressure, amytal, and mecholyl. Her blood pressure was 100/70 during the attack. When she was given neosynephrine, the systolic pressure increased to 140 mm Hg within 60 seconds and the rhythm reverted to normal. The second patient had an attack that had persisted for 20 hours in spite of the usual mechanical methods of treatment and the administration of mecholyl. The patient was given 4 doses of neosynephrine intravenously, 0.15 mg, 0.30 mg, 0.50 mg and 0.80 mg respectively, with ample time for the blood pressure to return to the preinjection level after each dose. The first 3 doses were ineffective. When the dose of 0.8 mg was given, the blood pressure rose from 110 to 160 mm of Hg and the rhythm reverted to normal 45 seconds after the beginning of the injection. The third patient had had the tachycardia for several hours before she was seen. She was given 0.4 mg of neosynephrine; the systolic blood pressure rose from 110 to 140 mm of Hg and the rhythm reverted to normal within the first minute after the injection. The fourth patient was a man who had had frequent attacks which did not stop spontaneously but could be stopped by the Valsalva maneuver. A dose of 0.5 mg of neosynephrine failed to restore the normal rhythm. A dose of 0.80 mg increased his systolic blood pressure from 90 to 130 Hg in 50 seconds at which time the rhythm reverted to normal.

Over-dosage with neosynephrine must be avoided because of the danger of producing an excessively high blood pressure. Occasional ventricular beats were seen with the larger doses used. However, experiments with animals have demonstrated that neosynephrine does not produce ventricular fibrillation even in relatively high doses in animals sensitized by cyclopropane anesthesia.[6] If a given dose is ineffective, a larger dose may be given after a 10-minute interval until the rhythm is reverted or until a rise of systolic pressure up to about 160 mm of Hg is produced. Doses between 0.5 mg and 1.0 mg should be effective in most cases.

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[1] Haney, H. F., Lindgren, A. J., Karstens, A. I., and Youmans, W. B., *Am. J. Physiol.*, 1943, **139**, 675.

[2] Youmans, W. B., Haney, H. F., and Aumann, K. W., *Am. J. Physiol.*, 1940, **130**, 190.

[3] Keys, A., and Violante, A., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 4.

[4] Keys, A., and Violante, A., *J. Clin. Invest.*, 1942, **21**, 1.

[5] Youmans, W. B., Gould, Jarvis, and Goodman, Morton J., experiments in progress.

Two of the 4 patients complained of mild precordial discomfort for a few minutes following the injection. This was the only unpleasant effect noted. Each patient described either a sensation of coolness of the skin or a tingling sensation evidently produced by the cutaneous vasoconstriction and piloerection.

Summary. Four nonhypertensive patients with idiopathic paroxysmal tachycardia of supraventricular origin were given neosynephrine intravenously in amounts sufficient to increase the systolic blood pressure 30 to 40 mm of Hg. In 2 of these the attacks had persisted for several hours in spite of all mechanical methods of treatment and administration of mecholyl. In all 4 cases the rhythm reverted to normal within one minute after the injection of neosynephrine. This is considered to be produced by reflexes from the carotid sinuses and aortic arch. Occasional ventricular beats occurred during the 2-minute period after the return to the normal sinus rhythm. Blood pressure returned to normal within 4 to 8 minutes after the injection.

Intravenous injection of neosynephrine or some other brief-acting vasopressor compound may prove to be the treatment of choice in selected cases of auricular or nodal paroxysmal tachycardia.

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[6] Meek, W. J., *Harvey Lect.*, Ser. XXXVI, 1940-41, 188.

15802

Dark-field Observations on Lymphocytes Exposed to X-Rays and Other Injurious Agents.*

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By the method of unstained cell counts, it has been shown[1,2] that X-rays had a delayed cytocidal action on lymphocytes irradiated *in vitro* and incubated at 37°C. The death of the cells was preceded by irregularities in the shape and the refractivity of the cells. In the present study, these morphologic changes were studied by dark-field illumination with a cardioid condenser.

On dark-field examination of a fresh suspension of the thymus of a rabbit, most cells appeared filled with fine intranuclear granules and a few cytoplasmic mitochondria (Fig. 1). After irradiation of the suspension and incubation for 3.5 hours, about 32% of the cells developed one or a few small, dark, round structures which may be termed primary vacuoles (Fig. 2). On dark-field examination, it was not possible to determine whether these structures were in the nucleus or cytoplasm. The vacuoles occasionally increased in size during observation. Some of them were surrounded by thick, bright, circular walls. They caused the distortion of the shape of many cells. They were present in viable cells which resisted staining with

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* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

[1] Schrek, R., *Radiology*, 1946, **46**, 395.

[2] Schrek, R., *J. Cell. and Comp. Physiol.*, 1946, **28**, 277.

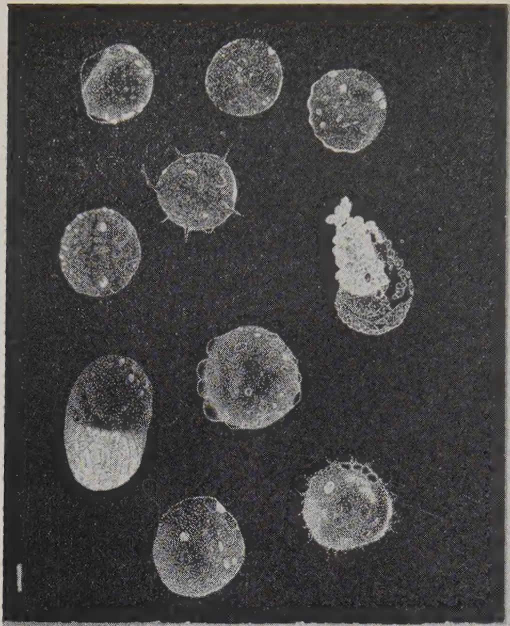
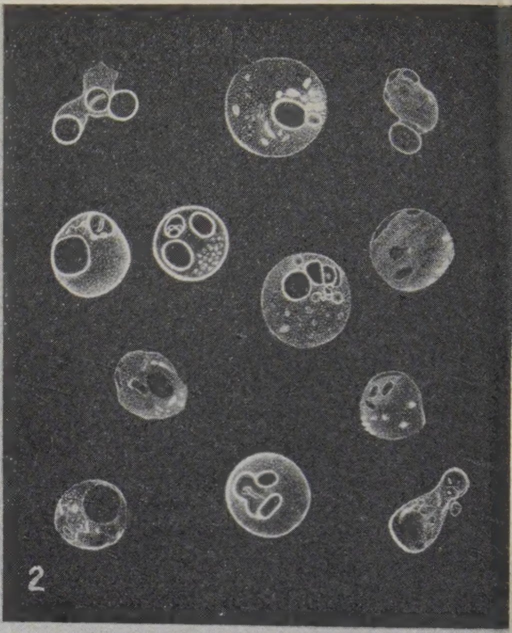
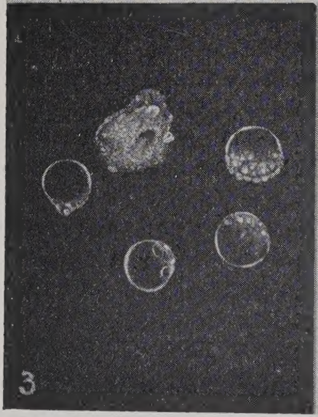
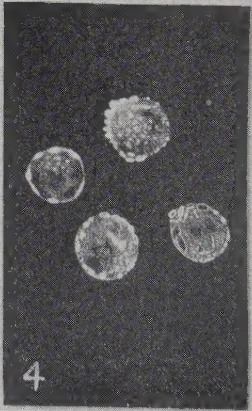
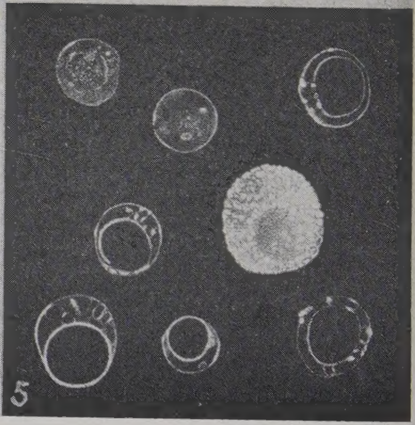

FIG. 1. Cells in a suspension derived from the thymus of a rabbit. The cells have innumerable fine intranuclear granules and a few larger mitochondria. A few cells show degenerative changes—small cytoplasmic bullae and thin cytoplasmic projections. Two granulocytes are shown.

FIG. 2. Thymic cell suspension, radiated (1000 r) and incubated for 3 hours at 37°C. The cells have one or more primary vacuoles, some of which are surrounded by bright vacuolar walls. A few cells are distorted.

FIG. 3. Thymic cell suspension, radiated (1000 r) and incubated 24 hours at 37°C. The cells are small, have relatively large secondary vacuoles and crescents of small and large granules. The cell walls are distinct. One normal granulocyte is present.

FIG. 4. Thymic cell suspension incubated for 3 hours at 45°C. The cells are small with fine and coarse granules.

FIG. 5. Thymic cell suspension treated with an equal volume of distilled water. The nuclei are dark, free of granules and have a distinct nuclear wall. The granulocyte appears normal.

Magnification in all figures is 1250 ×.

eosin. The spherical vacuolated cells had a greater average diameter than the nonvacuolated.

In histologic sections of the suspension, approximately 12% of the nuclei had large, acidophilic or slightly basophilic, Feulgen-

TABLE I.
A Comparison of the Characteristics of Primary and Secondary Vacuoles.

Method of study	Primary or early vacuoles	Secondary or late vacuoles
Dark-field examination		
Cell	Round or irregular Usually increased in size Usually no cell wall	Round Usually small Cell wall present
Vacuole	Single or multiple Small or large Usually have vacuolar wall	Single Relatively large No vacuolar wall
Histologic section	Intranuclear acidophilic area	Pyknotic nucleus
Method of unstained cell count	Usually in viable cells	In dead cells

negative vacuoles surrounded by dense chromatin material in the shape of a ring, horse-shoe or crescent. The intranuclear vacuoles in histologic sections correspond to the primary vacuoles observed on dark-field examination.

Similar vacuoles were observed both on dark-field examination and in histologic sections of rat thymic tissue excised 3 hours after irradiation *in vivo*.

On dark-field examination of a suspension irradiated *in vitro* and incubated 24 hours, 86% of the cells were small, had a thick, bright cell wall and a crescentic, polar mass of fine and coarse granules. Each of the cells also had a single, relatively large, round or oval, dark structure which was termed a secondary vacuole (Fig. 3). Cells with secondary vacuoles stained readily with eosin and were evidently dead. In stained sections, most cells had pyknotic nuclei which corresponded with the secondary vacuoles seen on dark-field examination.

A comparison of the primary and secondary vacuoles is given in Table I. It was usually possible to differentiate the 2 types of vacuoles on dark-field examination but intermediate forms were sometimes observed.

Nonirradiated suspensions incubated at 37°C also developed cells with primary and secondary vacuoles, but the rate of formation of the vacuolated cells was much slower. Apparently, irradiated and nonirradiated cells incubated at 37°C were killed by the same mechanism but irradiation accelerated the normal degenerative process.

In suspensions incubated at 45°C for 3 hours, the cells were dead and were filled with granules which appeared coarser and brighter than in the unincubated cells (Fig. 4). On histologic examination, many nuclei had large chromatin masses. The cells incubated at 45°C were apparently killed by a different mechanism (coagulation of protein?) than those incubated at 37°C. Furthermore, cytologic changes produced by heat were different from those induced by X-rays.

Irradiated cells incubated at 45°C appeared morphologically identical and survived as long as nonirradiated ones. Radiation did not accelerate the cellular degenerative processes at 45°C.

In view of Failla's[3] theory that X-rays cause the absorption of water into the nucleus, it is of interest to compare the effects of distilled water and of X-rays on lymphocytes. Addition of water to an equal volume of thymic cell suspension caused an immediate enlargement of the cells and a change in the nuclei which lost their granular appearance and became dark ill-defined areas (Fig. 5). Later the nuclear walls became visible as thin, bright lines. Histologic sections of thymic tissue incubated with water showed cells with vacuolated nuclei (Fig. 1 in Schrek[4]). In contrast, irradiated and incubated (37°C) cells in an isotonic solution suffered no morphologic change for 2 hours or more. After the latent period, there appeared small, dark, intranuclear vacuoles which enlarged and developed bright, vacuolar walls. Histologic sections showed vacuolated nuclei.

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[3] Failla, G., in Ward, H. B., *Some Fundamental Aspects of the Cancer Problem*, The Science Press, New York, 1937, p. 202.

[4] Schrek, R., *Am. J. Path.*, 1945, **21**, 1101.

From the reports in the literature and the findings in this laboratory, it is possible to formulate a hypothesis on the action of X-rays on lymphocytes. Presumably, the radiation produced, in one or more foci in the nucleus, first a photochemical change, then a chemical reaction which caused the absorption of water into these foci with the formation of primary vacuoles. The vacuoles enlarged forcing the chromatin material peripherally. The ring of chromatin ruptured at one point and then contracted resulting in a horseshoe shaped mass, a crescent and ultimately a small, round pyknotic nucleus.

Summary. Incubation of lymphocytes in suspension at 37°C produced primary and secondary vacuoles visible on dark-field examination. Preliminary irradiation of the suspensions greatly accelerated this process. The addition of distilled water to lymphocytes produced enlargement and vacuolization of the nuclei. The hypothesis is proposed that irradiation caused the absorption of water into one or more foci in the nucleus.